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2. The Influence of the total fuel-value of a dietary upon the quantity of Vitamine required to prevent Beri-beri. By W. L. BRADDON and E. A. COOPER. (*British Medical Journal*, Vol. I., 1914.)
3. The Factors concerned in the Solution and Precipitation of Euglobulin. By HARRIETTE CHICK. (*Biochemical Journal*, Vol. VII., 1913.)
4. The Viscosity of Protein Solutions. II. Pseudoglobulin and Euglobulin (Horse). By HARRIETTE CHICK. (*Biochemical Journal*, Vol. VIII., 1914.)
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6. The Precipitation of Egg-albumin by Ammonium Sulphate. A contribution to the theory of the "Salting-out" of Proteins. By HARRIETTE CHICK and C. J. MARTIN. (*Biochemical Journal*, Vol. VII., 1913.)
7. The preparation from animal tissues of a substance which cures Polyneuritis in birds induced by diets of Polished Rice. By E. A. COOPER. (*Biochemical Journal*, Vol. VII., 1913.)

8. On the protective and curative properties of certain Food-stuffs against Polyneuritis induced in Birds by a diet of Polished Rice. Part II. By E. A. COOPER. (*Journal of Hygiene*, Vol. XIV., 1914.)
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32. **On a remarkable new type of Protistan Parasite.** By H. M.
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LXIX. A STUDY OF THE BIONOMICS OF THE COMMON RAT FLEAS AND OTHER SPECIES ASSOCIATED WITH HUMAN HABITATIONS, WITH SPECIAL REFERENCE TO THE INFLUENCE OF TEMPERATURE AND HUMIDITY AT VARIOUS PERIODS OF THE LIFE HISTORY OF THE INSECT.

By A. BACOT, *Entomologist, Lister Institute of Preventive Medicine.*

(With Plates XXVII—XXXIV, 12 Charts and 3 Text-figures.)

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SECTION I. INTRODUCTION.

THIS research was undertaken at the instance of the Advisory Committee for the investigation of plague in India, its object and scope being to ascertain the effects of varying conditions of temperature and humidity on the species of fleas associated with rats and man: to trace if possible the critical temperatures and humidities which allow of or prevent their breeding, and further to ascertain if possible in which of the several stages of the flea's life history, whether as ova, larvae, pupae or imagines, the effects of extreme conditions of drought and heat proved most fatal. It was also expected that evidence might be forthcoming as to a probable aestivating or hibernating stage, in which these species could tide over periods when heat, drought or cold prevent active breeding and cause the flea population to fall to a minimum, or to disappear entirely, as in the case of *Ceratophyllus fasciatus* in the Punjab, between the months of May and November.

The methods and apparatus employed together with general remarks with regard to rearing, habits and life history of the flea, are treated as a separate chapter at the close of the introductory remarks. The experiments on breeding were of two kinds, firstly a series of rearing experiments from the egg onwards under different conditions, and secondly definite tests carried out separately on the several stages. The former as well as the latter have, however, been arranged under the various headings of eggs, larvae, cocoons and adults for the sake of convenience in tabulation. The numbers employed in each experiment in addition to the date should render the continuation of the individual experiments in the subsequent stages easy to follow.

The species used for these experiments were chiefly *Pulex irritans*, *Ceratophyllus fasciatus*, and *Xenopsylla cheopis*. Some work was also carried out with *Ceratophyllus gallinae* and *Ctenocephalus canis*, as well as with *Ctenocephalus felis* on one occasion, but difficulties in feeding these species on their proper hosts in captivity have prevented a plentiful supply of eggs being obtained, and restricted the scope of the experimental work. One set of experiments was also performed with *Leptopsylla musculi* in the summer of 1912, in order to study the conditions determining emergence from cocoons and the length of the resting period.

SECTION II. SPECIES AND ORIGIN OF THE FLEAS USED IN THE EXPERIMENTS.

Ceratophyllus fasciatus (Pl. XXVII, figs. 1 and 2). Some 30 adults were received from Prof. Minchin's stock (Lister Institute), which originated from fleas captured on rats trapped near Sutton Broad Laboratory, Norfolk, and a similar number were received from Prof. Nuttall's Laboratory at Cambridge.

Pulex irritans (Pl. XXVIII, figs. 1 and 2). A start was made with about a dozen specimens captured at Loughton and, although a few specimens were received from a friend in Hampshire, it is very doubtful if there is any infusion of blood from this source in the stocks used for the experiments, as the survivors of the specimens received were very feeble.

Ctenocephalus canis (Pl. XXIX, figs. 1 and 2), *Ctenocephalus felis* (Pl. XXIX, figs. 3 and 4) and *Ceratophyllus gallinae* are all of Loughton race.

Leptopsylla musculi (Pl. XXVII, figs. 3 and 4) were obtained from the Lister Institute.

Xenopsylla cheopis (Pl. XXVIII, figs. 3 and 4). Tubes containing living larvae of this species forwarded from India in the autumn of 1910 did not produce sufficient fleas to give an effective start, but specimens obtained subsequently by Dr Boycott from an English source have resulted in strong stocks, and the experiments have been performed with individuals of this race.

It is possible, even probable, that experiments on stocks from restricted localities may not give evidence of the full range of variation

of a species, which might enable it to adjust itself to the varying conditions of climate experienced in its geographical distribution. The known variation of habit in other insects, which meet needs called forth by slight differences within small areas, suggests this is likely to be the case¹. On the other hand the wide range of variation in duration of the cocoon period, found present in the stocks of *C. fuscatus* and *P. irritans*, would afford ample opportunity for selective action when taken in conjunction with their powers of rapid multiplication. It would be simple in the face of a climate with an extreme range of conditions, such as is experienced in some parts of India, for a race of *C. fuscatus* to be produced in which rapidly emerging individuals were in the majority at one period of the year and those having a long delayed period of emergence constituted the bulk of the flea population at a succeeding period. It seems probable that this is the explanation of the recorded complete disappearance of *C. fuscatus* during the hot months in the Punjab (*Journal of Hygiene*, Vol. VIII. No. 2, May 1908, p. 241).

In the case of *C. fuscatus* breeding cages were started in July 1910, but it was not until the autumn of that year that a sufficient stock was available for the production of ova in the numbers required, while with *P. irritans* it was only with the commencement of spring 1911 that egg laying was on a satisfactory basis. In consequence, the early experiments which deal with very small numbers but cover as wide a range of conditions as possible are to be regarded in the light of an attempt to ascertain the direction in which future experiments, with larger numbers, might most profitably be carried out. They also afforded an opportunity of improving the methods of treatment and feeding. Many of the results are of considerable interest and, as in the case of resting cocoons, are valuable in themselves, in spite of the small numbers of fleas employed; but others are unsatisfactory, if not deceptive, for the coincidence of food tests with seasonal changes renders it uncertain as to whether the results are due to one cause or the other.

There was no evidence of a definite resting phase during the earlier experiments but rather of a general variability dominated by temperature. With larvae of *C. fuscatus* taken from the cages during the

¹ E.g. *Papilio machaon* is single brooded in Norfolk and double brooded in Cambridge-shire.

Winter 1910-11, there was, however, a distinct tendency to rest in the cocoon stage, and this was in great measure irrespective of the range of experimental conditions.

In one instance broods were ruined owing to a too drastic reduction in the humidity of an incubator; in another to the use of a sample of sand which proved inimical to the larvae; the cause of this was suspected to be the presence of sodium chloride.

It has been thought best to include all the experiments without exception, however clear the cause of failure might appear, in the hope that future workers in this new field of research may find the results of some value.

SECTION III. APPARATUS AND EXPERIMENTAL METHODS EMPLOYED.

(a) Range of external conditions; temperature and humidity.

The experiments were carried out chiefly in four incubators, two of which were maintained at 75° F.¹, but with differing degrees of humidity, and a similar pair at 85° F.

In addition use was made of a cellar, the Laboratory cupboard and one situated next to a chimney flue, the fire being unused between the months of May and September.

Since December 1910 use has also been made of an empty beehive in the garden.

INCUBATORS. For clearness the incubators have been designated by their temperature and their condition as regards humidity, thus: incubator 85 Wet; incubator 85 Dry; incubator 75 Wet; incubator 75 Dry. Records of temperature and humidity were taken twice daily, except on Sundays, when as a rule only one reading was made. These are set out in tabular form below, pp. 460-2, and graphically shown in Charts 1 and 2. A few records had to be discarded owing to the Wet bulb thermometers being out of order. The conditions aimed at were to keep one at 85° F. with humidity at .70; one at 75° F. humidity .75; one at 85° F. humidity at .55 to .60, and one at 75° F. with humidity at .50 to .55.

The degree of humidity in the air is according to Glaisher—"The ratio of the quantity of vapour present in any volume of the air to

¹ All temperature readings are on the Fahrenheit scale.

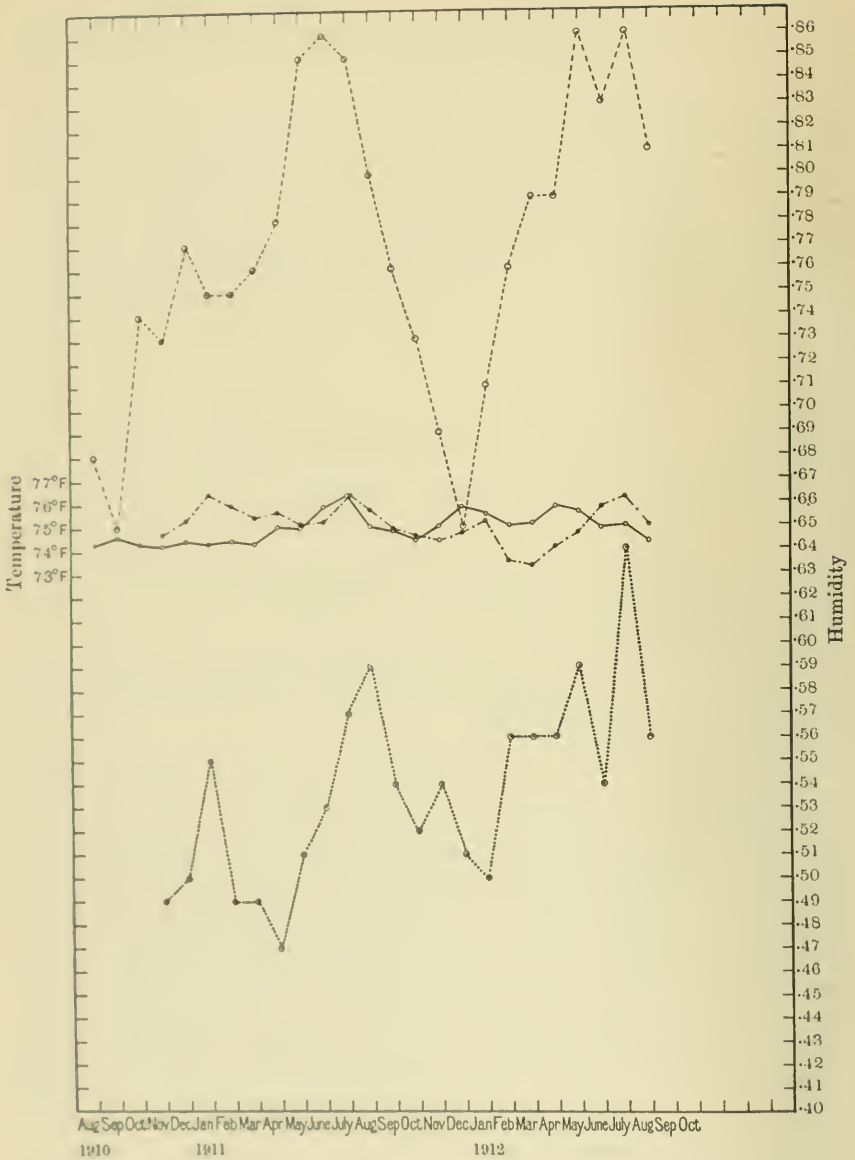


Chart 1.

Incubator 75 Wet.
Incubator 75 Dry.

Temp. ————— Humidity - - - - -
Temp. - . - . - . - Humidity

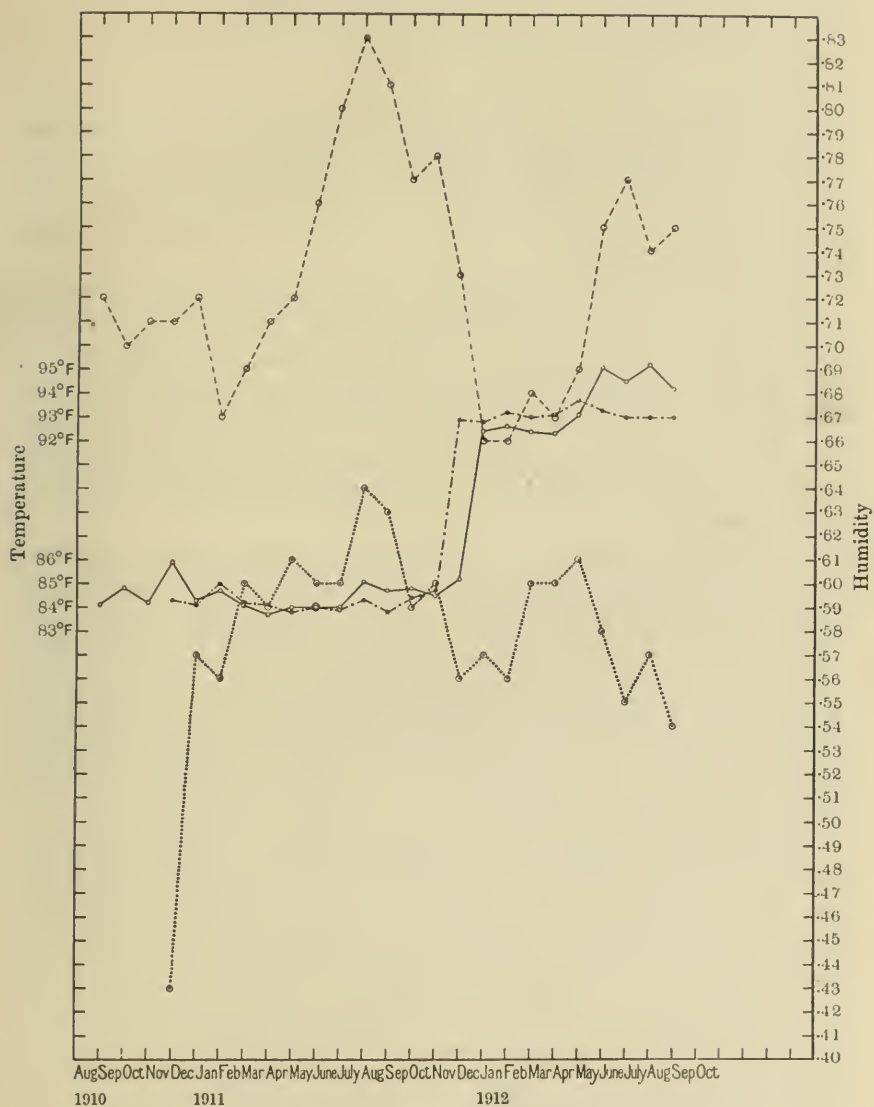


Chart 2.

Incubator 85/95 Wet.
Incubator 85/95 Dry.

Temp. ————— Humidity -----
Temp. Humidity

the quantity which would have been present in the same volume, had the air been completely saturated." The readings of the "Dry" and "Wet" bulb thermometers were taken and the percentage of humidity obtained from Glaisher's tables.

During November 1911 the temperature of the two hot incubators, 85 Wet and 85 Dry, was raised to 93° F. or thereabouts, as it was desired to make certain tests under conditions of greater heat; from the time of change onwards they are referred to as 93 Wet and 93 Dry—a few experiments that had been started at the lower temperature being finished at the higher.

Except during the hot summer and cold winter months, it will be seen that the averages are roughly in the neighbourhood of the desired figures. From the contrasted breeding experiment dealing with *P. irritans*, *X. cheopis* and *C. fasciatus* (Table XXV) it will be seen that the rise in humidity of 85 Dry and 75 Dry to '63 and '59 respectively in these two incubators was not sufficient to permit of any larvae surviving, so that the experiments were not really interfered with from this cause.

LABORATORY CUPBOARD. (Chart No. 3.) This is a rather cool and very draughty place with a cement floor, on a side of the building that never gets any direct sunshine on it. Its temperature tends to be rather lower than that of a room without a fire and its humidity during cool wet autumn or winter weather is well in excess of the incubators; it tends to be drier than the incubators during the spring and summer months. A glance at the experiments (Tables XVII and XXI) will show that, while the conditions are next to impossible for *C. fasciatus*, they are successfully surmounted by a percentage of *P. irritans*.

THE CELLAR. (Chart No. 4.) This gives an even range of temperature seldom differing by more than one or two degrees from night to day, and ranging from an average of 44° F. in February to 64° F. in August 1911 (four degrees higher than the summer temperature of the previous year). Its record of humidity is even more stable—being practically constant at '91 to '93. It has a bricked but uncemented floor and probably represents fairly well the conditions of cellarge prior to the free use of cement in modern buildings.

The conditions were found to be very favourable to all stages of the flea's life history with the exception of egg laying, and possibly hatching—save during the warm months—but development of the larvae is slow, so slow in some instances that it is a question whether the final failure of larvae to spin, as in experiment 6th October 1910 with

P. irritans, may not have been due to their food deteriorating during the long delay and their ultimate starvation.

WARM CUPBOARD. (Chart No. 5.) As will be seen from the tables, the range of conditions is not very wide, apart from the extremes of the summer of 1911. Normally the temperature is between 60° F. and 70° F. and its humidity not far from .55 to .60. Its conditions seem to be even less favourable for the hatching of eggs than the low humidity incubators 85 Dry and 75 Dry, and at least equally fatal to larval development.

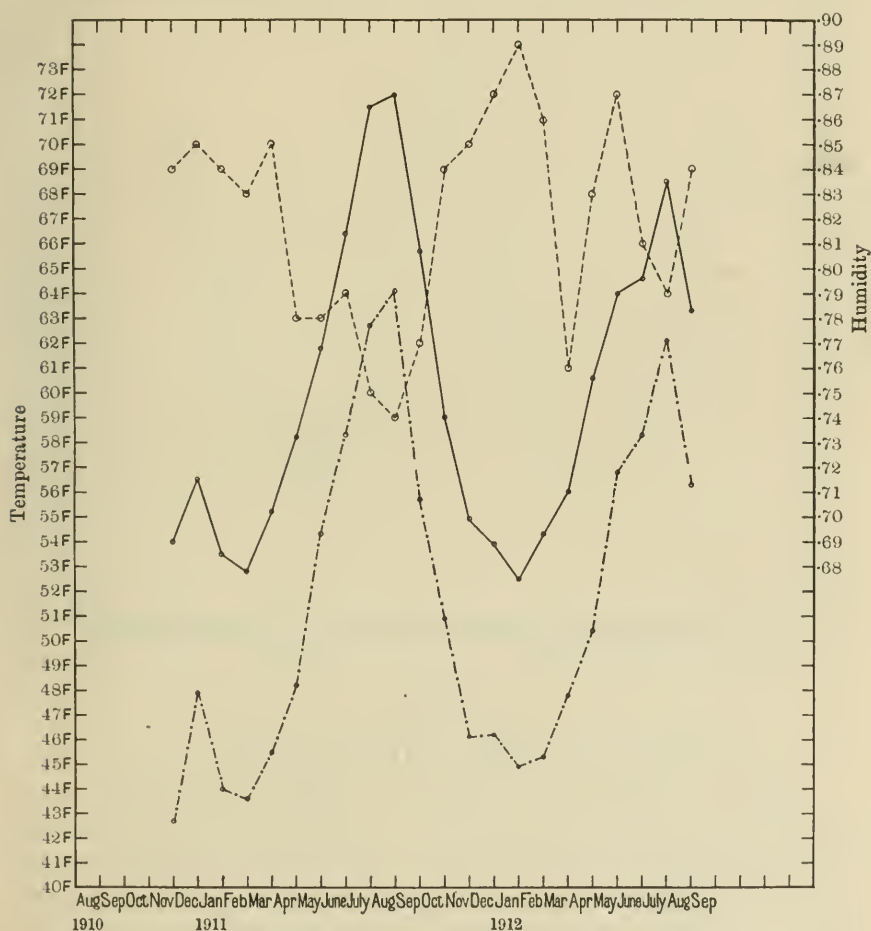


Chart 3.

Laboratory Cupboard. Temperature Max. ————— Humidity -----
Min. - - - - -

Full records of temperature and humidity were not kept for the cupboards and cellar until the autumn of 1910; there is also a gap from April to July 1912, when the readings of "Wet" and "Dry" bulb thermometers were not taken in the cellar, as the latter instrument was in use elsewhere. The previous record, however, over a long period, and that for the months of August and September 1912, had remained constant so that the estimate of '93 is considered near enough for all practical purposes.

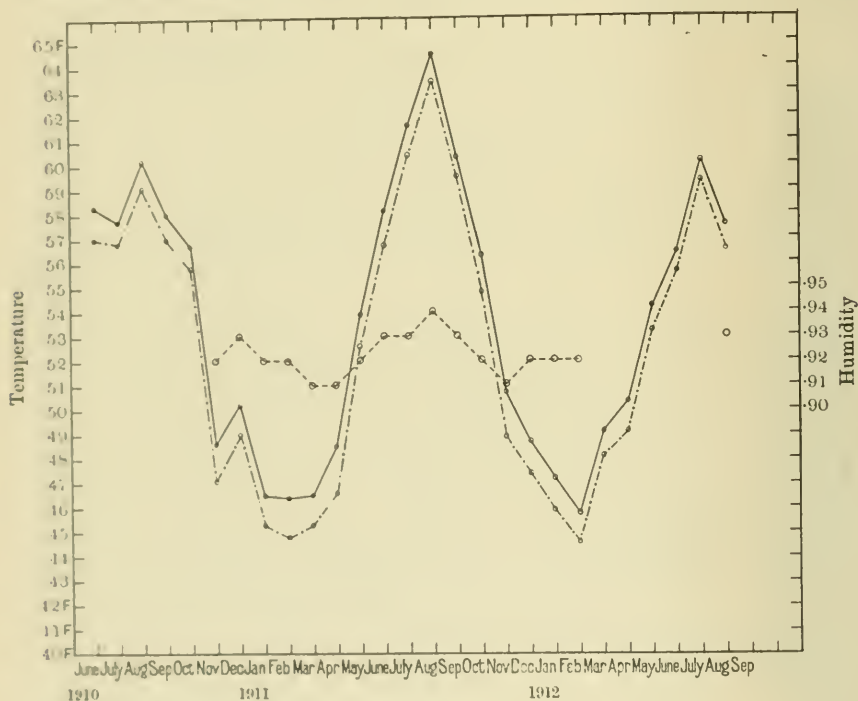


Chart 4.

Cellar. Temperature Max. ————— Humidity - - - - -
 Min. - - - - -

BEEHIVE. (Chart No. 6.) This was installed as a control to test the influence of the natural extremes of night and day temperatures, chiefly with regard to the cocoon stage. No humidity records were taken as it was thought that these were unlikely to yield trustworthy comparison with the conditions elsewhere unless taken at frequent intervals. As might be expected, the results obtained by its use varied according to the weather conditions prevailing, but evidence is afforded that

the larvae of some species can withstand considerable cold, even when newly hatched.

In order that the records of humidity might be as accurate and reliable as possible, Dr Martin kindly devised an air circuit past the wet bulb thermometers in the incubators in order to guard against the

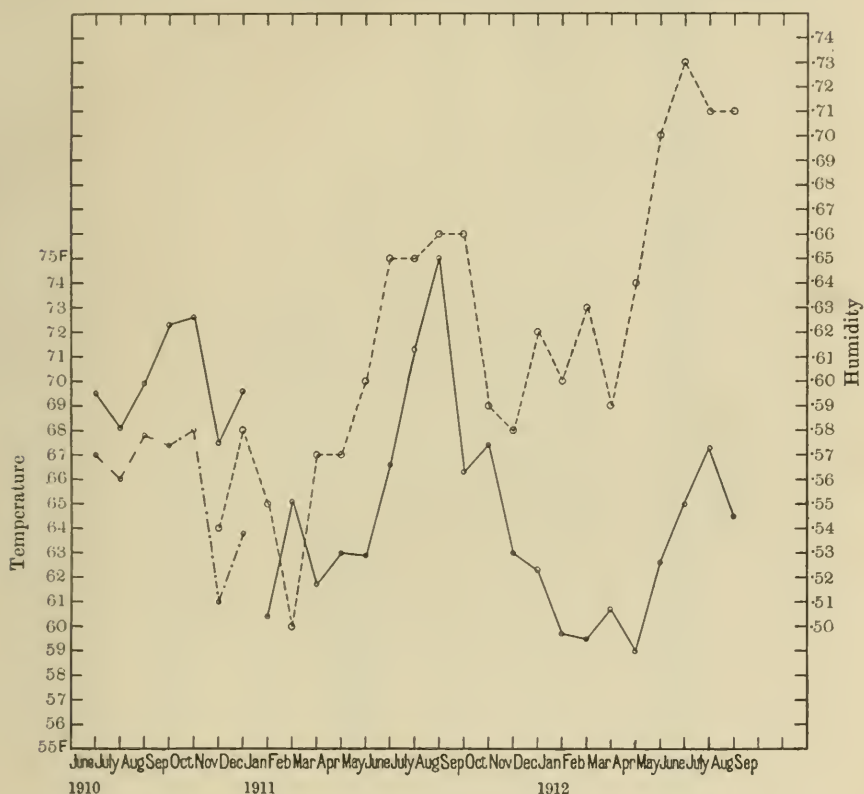


Chart 5.

Warm Cupboard. Note up to Dec. 1910 Max. and Min. records were made, after that date readings are of the "dry" bulb of the Hygrometer.

Temperature Max. — After Dec. 1910 Dry bulb of Hygrometer —
Min. - - - - - Humidity - - - - -

error involved when readings are taken in a nearly still atmosphere. The readings were in every case the minimum obtained after working the air circuit illustrated on p. 459. There still remains, however, the fact that the mortality in such a place as a house cupboard is far higher in comparison than in the incubators. It is suggested that this is

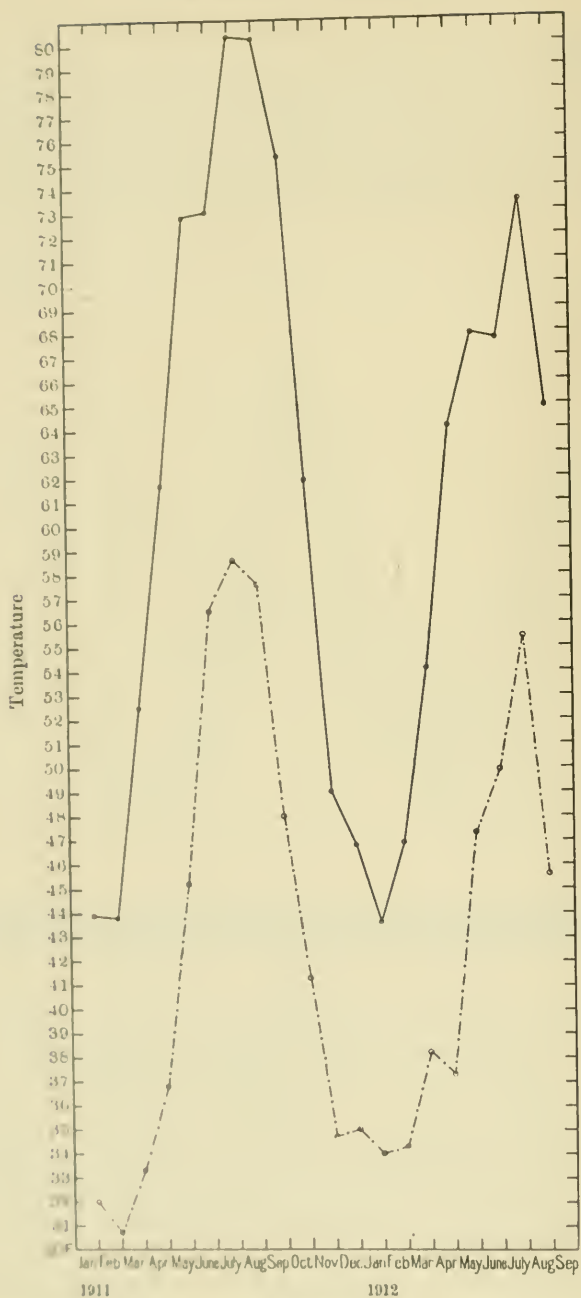


Chart 6.

Bechive. Temperature Max. — Min. - - - - -

due to the inequality of the humidity in the latter. The small amount of current in an incubator possibly allows of "pockets" of moist air to remain undisturbed in the receptacles in which the larvae are reared. Possibly the moisture given off by the larvae themselves may produce an envelope of humid air which enables them to escape the desiccation that would inevitably occur in a more draughty situation¹.

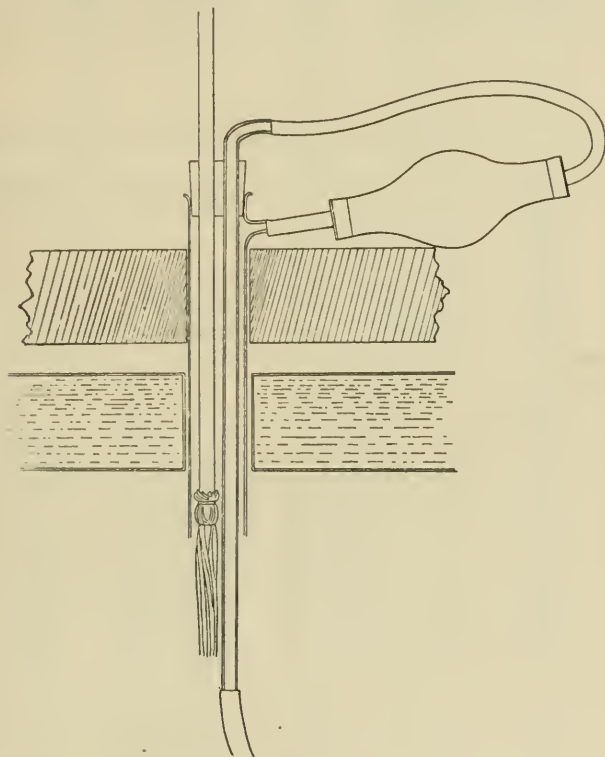


Fig. 1.

In contrasting results this possibility should be borne in mind, as it seems possible that all the incubator humidities should be regarded as higher than the actual readings, in comparison with other situations. Even with incubators having a wide range of ventilation adjustment and a water tray subdivided so as to allow of a varying area of exposed

¹ For example, in the case of the newly hatched larvae trials in incubator 75 Wet (see Table X); the average length of life is only half as long in July as during the cold months. This is apparently due to a less stagnant atmosphere in the former case. It is usual to keep the ventilator more widely open during the summer as this can be done without any corresponding fall in humidity.

water surface, it is impossible, during the summer, to maintain a steady percentage of moisture in the air for more than a few hours or days together, as all the conditions are swayed by external changes. In the winter and autumn, especially at night, when a clearing sky results in a ground frost, there is a rapid fall in the amount of moisture in the outside air drawn into the incubators, and when hard frosts set in it is difficult to maintain sufficient humidity to enable the larvae to escape destruction, even with the ventilators shut down and wet cloths hung in the interiors.

Incubators. Monthly Averages of Humidity and Temperature.

Month 1910	Incubator Temp.	85° Wet Hum.	Incubator Temp.	75° Wet Hum.	Incubator Temp.	85° Dry Hum.	Incubator Temp.	75° Dry Hum.
Aug.	84.1	.72	74.3	.68				
Sept.	84.8	.70	74.6	.65				
Oct.	84.2	.71	74.3	.74				
Nov.	85.9	.71	74.2	.73	84.3	.43	74.7	.49
Dec.	84.3	.72	74.4	.77	84.1	.57	75.3	.50
1911								
Jan.	84.7	.67	74.3	.75	85.0	.56	76.4	.55
Feb.	84.1	.69	74.4	.75	84.2	.60	75.9	.49
March	83.7	.71	74.3	.76	84.1	.59	75.4	.49
April	84.0	.72	75.0	.78	83.8	.61	75.6	.47
May	84.0	.76	74.9	.85	84.0	.60	75.1	.51
June	84.0	.80	75.8	.86	83.9	.60	75.2	.53
July	85.1	.83	76.3	.85	84.3	.64	76.3	.57
Aug.	84.7	.81	75.0	.80	83.8	.63	75.7	.59
Sept.	84.8	.77	74.8	.76	84.4	.59	74.9	.54
Oct.	84.5	.78	74.4	.73	84.7	.60	74.6	.52
Nov.	85.2	.73	75.0	.69	92.9	.56	74.4	.54
Dec.	92.4	.66	75.8	.65	92.8	.57	74.7	.51
1912								
Jan.	92.6	.66	75.5	.71	93.2	.56	75.2	.50
Feb.	92.4	.68	75.0	.76	93.0	.60	73.5	.56
March	92.3	.67	75.1	.79	93.1	.60	73.3	.56
April	93.1	.69	75.8	.79	93.7	.61	74.1	.56
May	95.1	.75	75.6	.86	93.3	.58	74.7	.59
June	94.5	.77	74.9	.83	93.0	.55	75.8	.54
July	95.2	.74	75.0	.86	93.0	.57	76.2	.64
Aug.	94.2	.75	74.3	.81	93.0	.54	75.0	.56
Sept.								

(Readings twice daily.)

NOTE. A 3 Nov. 1910 20 Temp. and 14 Humidity readings only.

F 3 „ 20 „ 14 „ „ „

Laboratory Cupboard.

1910	Number of readings	Temperature		Humidity
		Max.	Min.	
Nov.	23	54.0	42.7	.84
Dec.	31	56.5	47.9	.85
1911				
Jan.	31	53.5	44.0	.84
Feb.	28	52.8	43.6	.83
March	31	55.2	45.5	.85
April	30	58.2	48.2	.78
May	31	61.8	54.3	.78
June	30	66.4	58.3	.79
July	31	71.5	62.7	.75
Aug.	31	72.0	64.1	.74
Sept.	30	65.7	55.7	.77
Oct.	31	59.0	50.9	.84
Nov.	30	54.9	46.1	.85
Dec.	31	53.9	46.2	.87
1912				
Jan.	31	52.5	44.9	.89
Feb.	29	54.4	45.3	.86
March	31	56.0	47.8	.76
April	30	60.6	50.4	.83
May	31	64.0	56.8	.87
June	30	64.6	58.3	.81
July	31	68.5	62.1	.79
Aug.	31	63.3	56.3	.84
Sept.				

Cellar.

1910	Number of readings	Temperature		Humidity
		Max.	Min.	
May	19	56.0	54.1	
June	28	58.3	56.9	
July	31	57.7	56.8	
Aug.	30	60.2	59.1	
Sept.	30	58.0	57.0	
Oct.	30	56.7	55.8	
Nov.	30	48.6	47.1	.92 (27 readings of humidity)
Dec.	30	50.2	49.0	.93
1911				
Jan.	31	46.5	45.3	.92
Feb.	28	46.4	44.8	.92
March	31	46.5	45.3	.91
April	30	48.5	46.6	.91
May	31	53.9	52.6	.92
June	30	58.1	56.7	.93
July	31	61.6	60.4	.93
Aug.	31	64.5	63.4	.94
Sept.	30	60.3	59.5	.93
Oct.	31	56.3	54.8	.92
Nov.	30	50.7	48.9	.91
Dec.	31	48.7	47.4	.92
1912				
Jan.	31	47.2	45.9	.92
Feb.	29	45.8	44.6	.92
March	31	49.1	48.1	.93 (estimated)
April	30	50.3	49.1	.93 (estimated)
May	31	54.2	53.2	.93 (estimated)
June	30	56.4	55.6	.93 (estimated)
July	31	60.1	59.3	.93 (estimated)
Aug.	31	57.5	56.5	.93 (27 readings of humidity)
Sept.				

*Bionomics of Fleas**Warm Cupboard.*

1910	Number of readings	Temperature		Humidity
		Max.	Min.	
May	18	71·9	67·1	
June	28	69·5	67·0	
July	31	68·1	66·0	
Aug.	31	69·9	67·8	
Sept.	30	72·3	67·4	
Oct.	30	72·6	68·0	
Nov.	30	67·5	61·0	·54 (27 readings of humidity)
Dec.	28	69·6	63·8	·58 (31 readings of humidity)
1911				
		Dry Bulb		
Jan.	29	60·4		·55
Feb.	27	65·1		·50
March	31	61·7		·57
April	29	63·0		·57
May	30	62·9		·60
June	30	66·6		·65
July	28	71·3		·65
Aug.	30	75·0		·66
Sept.	30	66·3		·66
Oct.	29	67·4		·59
Nov.	30	63·0		·58
Dec.	30	62·3		·62
1912				
Jan.	31	59·7		·60
Feb.	29	59·5		·63
March	31	60·7		·59
April	30	59·0		·64
May	31	62·6		·70
June	30	65·0		·73
July	31	67·3		·71
Aug.	31	64·5		·71
Sept.				

Beehive.

1910	Number of readings	Temperature	
		Max.	Min.
Dec.	3	50·0	29·0
1911			
Jan.	31	43·9	32·0
Feb.	28	43·8	30·7
March	31	52·5	33·3
April	30	61·7	36·8
May	31	72·8	45·2
June	30	73·0	56·5
July	30	80·3	58·6
Aug.	31	80·2	57·6
Sept.	30	75·3	48·0
Oct.	31	61·9	41·3
Nov.	30	49·0	34·7
Dec.	31	46·8	35·0
1912			
Jan.	31	43·6	34·0
Feb.	29	46·9	34·3
March	31	54·1	38·2
April	30	64·1	37·3
May	31	67·9	47·3
June	30	67·7	49·9
July	31	73·4	55·4
Aug.	31	64·9	45·6
Sept.			

(b) General arrangement and management of breeding cages.

For rearing rat fleas I have used a slightly modified form of the cage designed for the infection experiments of the Advisory Committee illustrated in the *Journal of Hygiene*, Vol. VI. No. 4, Sept. 1906, Plate IV. The alterations consist of somewhat enlarging the cage and making the inner cage larger in proportion to the outer. Several different sizes have been tried, the most convenient is: Outer glass cage 10" × 10" × 18", inner wire cage 8" × 8" × 8", neck or entrance tube 5" in diameter by 3" deep. The pan 9" × 9" × 1" deep. Copper or brass wire gauze is preferable to iron as it does not rust. In place of the tin lid a wire gauze cover keeps the cage drier and sweeter, and a muslin cover¹ may be used over this to ensure that no fleas escape. The cover of the outer cage is of muslin tied round the neck or entrance tube and fastened to the glass sides with glue. It is well to use some cement that can be softened, as portions of the cover have to be removed occasionally. The floor, pan and all, should be covered with about one to one and a half inches of silver sand or sawdust. Sand should be washed and then baked to make certain that it neither contains any salt nor harbours mites or other harmful organisms.

A single large rat, or two small ones, may be kept in a cage of this size without its getting too humid under normal atmospheric conditions. Occasionally, say at monthly intervals, a quantity of the sand and the rats' bedding should be shifted to the space between the wire cage and the glass and returned the following month. Under exceptional circumstances it is necessary to remove a portion of the sand and bedding entirely, in order that it may be slowly dried in some deep walled receptacle; it may then be returned with its fauna of larvae and fleas.

In cages for breeding *C. fasciatus* it is well to supply the rat with some pieces of cloth or felt as bedding; this very much facilitates the collection of both fleas and their larvae which will be found wandering over and through the felt respectively.

As regards *X. cheopis*, cloth bedding for the rat does not offer the same advantages for obtaining fleas and their larvae. The former are usually to be found in the greatest numbers on the rat itself or in the sand, while the larvae are usually distributed through the sand and are seldom seen on the cloth. Cocoons are, however, to be obtained in numbers spun on buried or partially buried pieces of cloth placed between the outer and inner walls of the cage.

¹ The best material to employ is what is known by drapers as "Nainsook."

FOOD FOR THE RATS. Bread and milk, given in small pans, was the only food used in the cages during the first year. It was adopted in preference to grain, bran, etc., as a precaution against mites, which are frequently present in corn chandlers' stores. A better method adopted later is to give some oats, a piece of new bread and a drink of milk daily.

With mice I am informed that the proper diet is mixed oats and hemp seed and a small piece of bread which, after being wetted and squeezed to remove the superfluous moisture, is left in the cage for an hour or so and then removed. This method has proved quite successful for mice, but for rats more liquid is essential.

The grain and seed should be heated before use to kill off any mites or other organisms it may harbour. Although we were able to keep our cages free from mites for many months, they eventually became infected, possibly by way of the animals when changes took place. At least two species were present—the more numerous being one of the vegetable-feeding Tyroglyphidae that is very commonly associated with grain stores. The other was a species of *Cheyletus* closely allied to, if not identical with, *Cheyletus eruditus*. This mite probably lives chiefly on the vegetable feeding species, but will undoubtedly attack and destroy flea larvae, a specimen having been caught in the very act.

It is questionable, however, if this species is very harmful in the cages when the other mites are numerous, as the latter will be much easier prey for the *Cheyletus* than the frantically active flea larvae. When the flea larvae are moulting, the case may be otherwise, and, should the *Cheyletus* be present to a large extent, or the vegetable feeding species of mites be absent or present in small numbers only, serious mortality of the flea population might occur.

(c) *Methods employed in rearing fleas and experimenting with them.*

1. OBTAINING AND HATCHING EGGS. To obtain ova of *Pulex irritans*, the adults are kept in one-inch cardboard boxes with glass bottoms¹,

¹ In each incubator or other place used for experiment a jar of sand is kept in which are buried the boxes containing fleas for egg laying or cocoons awaiting emergence and the tubes of adult fleas for longevity tests. Glass tubes containing newly hatched larvae whose length of life without food is being determined should be partially buried in the sand to prevent any condensation of moisture in the tube.

This method was instituted so as to render the conditions of the experiments on adults more natural, the habit of the flea being to hide away in crevices, corners, etc., where more equable conditions of temperature and humidity are likely to obtain than elsewhere.

The result proved satisfactory, as the mortality among the fleas put into boxes for egg laying was greatly reduced, and the average number of eggs laid was increased.

the lid replaced by a cover of fine silk gauze (chiffon) securely tied on. A loose ring of dark cloth is placed round the inside of the box to give the fleas foothold, and this also proves very convenient for the removal and counting of the eggs that are laid on it. If the card of the box is stained with methylene blue or other dark colour, the finding and counting of those eggs that are laid off the cloth are facilitated. This box is nested in one of the next larger size for security and only removed for feeding, when the gauze is placed against any convenient skin surface. One quarter of an hour per day is sufficient time for feeding, but larger numbers of eggs are laid in response to more food. The fleas must be shifted to a fresh box or the ova removed every three or four days to prevent any larvae that may be hatched escaping through the mesh of the gauze.

Rat, dog or other fleas are collected from the host or its bed and put into boxes. Up to 50 females and the due proportion of males are put into a one-inch glass-bottomed box, and for greater numbers a box of one and a quarter inch diameter is used.

The box is prepared in the same way as for *P. irritans*, but the gauze cover may be omitted (for method of boxing fleas, see p. 464). The boxes containing the fleas are buried in the sand pots mentioned in footnote on p. 464. To induce the fleas to lay freely the boxes are kept in a humid atmosphere at a temperature of 70° to 75° F. The fleas are left in the boxes for 24 hours and then returned to the cages.

It is not so necessary to bury *P. irritans* as it is *C. fasciatus* and *X. cheopis*, but in the hotter incubators or during cool weather it is advisable. *X. cheopis* does not lay freely unless the cage containing both fleas and their host is kept at about 70°—75° F.

HATCHING. Eggs are best left where they are laid if this is possible, for, though they can usually be moved by the use of a moistened camel's hair brush with safety, it is not always possible to avoid injuring them, as they occasionally become quite firmly attached in spite of the fact that no cement is used by the female when they are laid.

The powers of abstinence on the part of the newly hatched larvae renders it practicable to leave the hatching box without food supply; the young larvae are then collected day by day by inverting the box in the mouth of a glass tube, when, after gently tapping, they fall into the bottom of the tube. A warm humid atmosphere is best to ensure a speedy emergence from the egg. Cool conditions lower, and hot and dry ones raise, the percentage of failures without proving so absolutely fatal to the ova as they are to the larvae.

2. FOOD AND TREATMENT OF LARVAE. Glass tubes, entomological boxes and card jars have been used as receptacles for rearing larvae. Glass tubes one and a quarter to one and a half inches in diameter give good results for small numbers, but they are liable to condense moisture if the air is humid or subject to large temperature variations. Glass-bottomed boxes did not give satisfactory results and are not very convenient, as with humid conditions the cardboard lids swell and become too tight. "Mono Service" card cream jars are light, unbreakable, and being made of compressed waxed card, are not absorbent, and at the same time do not condense moisture so readily as glass. Most of the experiments were carried out in half-pint jars of this description. A cover of thin muslin, fastened by an elastic band, served to keep out dust and prevent any rapidly developed fleas from escaping without seriously checking free interchange of temperature and humidity.

Food (for a general discussion on the food of flea larvae, see Section IV, subsection 2).

In the experimental work five separate diets were tried:—

(1) Blood-soaked rag, which is first to be recommended. Any slaughter-house can supply a mutton-cloth dipped in blood, and if this be wrapped in paper and put in a dry situation it will keep indefinitely and may be cut into snippets as required. This formed the chief food used in the experiments, and is designated, for brevity, B.S. Rag.

(2) Flea faeces, in which case the gauze covers to boxes in which fleas have been fed, are very convenient provided they are well spotted and blotched with the blood and dejecta voided during and subsequent to feeding. This food, referred to as "flea faeces," although the blood ingested was human, seemed to suit *C. fasciatus*, *X. cheopis* or *P. irritans* equally well.

(3) Dead flies. A few experiments were made with *Musca domestica* and *P. irritans*. The diet proved a moderate success, but required renewing constantly, and the time expended in catching flies, together with shortage of supplies in late autumn, prevented further trials. The fact that the cocoons are very difficult to find when flies are used makes this food quite unsuitable for some sections of experimental work.

(4) Bran gave very poor results for feeding *C. fasciatus* when used in the small quantities that it is convenient to work with if close observation is required before the fleas actually emerge. It is also open to the same objection as the dead flies as regards the difficulty of finding the cocoons.

(5) Rat Faeces. These gave very poor and unsatisfactory results with *C. fasciatus*. Crushing or chopping the faeces was tried and improved matters somewhat. Larvae in some instances fed well, and the percentage of cocoons was high, but the actual numbers of *C. fasciatus* reared were small. Larvae of *P. irritans* and *X. cheopis* succeeded much better on this food, which is a decided improvement on the flies and bran although the cocoons are not easy to find. Possibly under natural conditions, when a fresh supply is continually available, rat faeces may be a more important article of diet than the experimental evidence suggests.

COVER FOR THE LARVAE. A small quantity of fine sand or other finely divided material should be placed at the bottom of the receptacles used for rearing larvae; about $\frac{3}{4}$ of an inch is sufficient, the food being mixed in with the sand. It is not necessary to use sand with bran, dead flies, or some other foods, and larvae have been reared on dried blood alone in a glass tube. Its use is, however, a necessity when rearing in a cage, as pointed out in the Reports of the Plague Commission (*Journal of Hygiene*, Vol. VIII. Chap. XXIX. Section 1), and is a convenience when rearing apart from the host, as it renders the finding of cocoons and their removal much easier. The sand or other material used ought always to be dry enough to fall grain by grain; it is detrimental when wet enough to cling.

HUMIDITY. When rearing in very dry places a local humidity may be attained by placing at the bottom of the receptacle a disc of blotting-paper with a tag projecting above the sand; this tag can be wetted at intervals. The method of placing damp blotting-paper *above* the sand or a wet muslin cover to the tube or jar may be pursued. The sand itself should on no account be damped. The adjustment of this moistening, having regard to the local conditions of temperature, humidity and air circulation, makes all the difference between 100% mortality in the first two days and the successful rearing of adult fleas, but too much or too little moisture may be equally fatal (compare tables and note the effect of moistening in incubator 75 Dry and 75 Wet, also between 85 Dry and 85 Wet).

Urine, as compared with distilled water, appears to be a more effective damping agent. Possibly the salts it contains retain the moisture and develop a pocket of moist air in the receptacle used, while with distilled water the desiccation is more complete. While admitting that the evidence for this last opinion is none too clear, there seems no doubt whatever as to the main issue of the beneficial effects of moistening generally in a dry atmosphere.

METHODS OF HANDLING. To collect individual larvae from the dust and rubbish of an animal's bed or nest is a slow task. By using a series of wire or muslin sieves the process may be much quickened; an ordinary wire document basket makes an excellent sieve for the first stage; small boxes with the bottoms replaced by fine mosquito netting and fine veil netting can then be used, only fine dust, larvae and eggs will be left in the pan. Much of the fine dust can afterwards be gently blown away. The best method of collecting larvae from dust or sand that is too heavy to be disposed of by winnowing is to spread the sand or dust thinly over a black or dark-coloured card and pick the larvae out with a fine moistened sable or camel's hair brush. Flea-larvae are usually very conspicuous, for such small objects, owing to their frantic activity, but occasionally may be missed as they lie still in a stiff stretched-out position or coiled up watch-spring fashion. Under these circumstances they are difficult to see, especially when small and coiled. It is best to leave the sand undisturbed for a few minutes and then re-examine, as they usually renew their activity after a short rest.

To test the length of life without food of freshly hatched larvae they were placed in small corked glass tubes, three-eighths of an inch by two inches, with a few scraps of blotting-paper to afford them foothold and cover. Coloured blotting-paper renders observation and counting easier. A subsequent method was to use a little fine sand and to plug the tube with cotton-wool; under these circumstances it is necessary to keep the tube upright.

3. **OBTAINING AND TREATMENT OF COCOON STAGE.** In treating the cocoon as a definite stage, convenience alone has been followed.

To obtain cocoons for experiment, full sized larvae are sorted out from the cages, breeding jars or the host's bed as the case may be. They are placed in a receptacle containing fine sand or dust and a supply of food and kept in a humid atmosphere at 70°—75° F. The cocoons must be removed daily from the receptacle if their subsequent history is to be at all closely followed; they are therefore removed to a one-inch glass-bottomed box and buried in the sand pots. For as the cocoons are naturally buried beneath dust or litter, or spun in crevices, it is an obvious precaution to bury the box in which they are kept if fair results are to be obtained.

4. **CONTROL AND TREATMENT OF ADULTS. TRANSFERENCE OF FLEAS.** For shifting, transferring or sorting fleas the use of a large pan with a white glazed surface, as recommended in the report of

the Advisory Committee (*Journal of Hygiene*, Vol. VIII. No. 2, p. 256, May 1908), was adopted.

For dealing with *P. irritans* a much deeper pan is necessary than for the rat fleas; it must also be wide, as otherwise the hands and arms of the operator prevent a clear view of the bottom being obtained. An enamelled iron "chef" bowl is suitable, but it should be ten inches to a foot deep, for *P. irritans* can jump at least a foot even from the slippery take-off afforded by an enamelled pan, and experience has shown that it can jump on to the clothing of a person stooping over the pan although unable to clear the rim. *Ct. canis* and *C. gallinae* are also good jumpers and require a deeper pan than either of the rat fleas. The jumping habit soon falls into abeyance, however, when the fleas are kept in small boxes and fed.

All jars, tubes or boxes in which larvae are being reared or cocoons kept, should be opened after being placed on the bottom of the pan, and in changing from box to box the same precaution ought to be followed. To collect fleas from the pan, an ordinary test-tube and a small camel's hair brush are all that is required. If the test-tube be held with its mouth in front of the flea, tilted at a slight angle so that the upper rim is above the insect, it will usually hop into the tube; the brush may be used to expedite matters or to aid refractory specimens to take a right direction. In case it is desired to collect *P. irritans* in boxes, the fleas being already in the pan, the box with cloth lining ready for their reception is dropped mouth downwards over first one and then another; each flea covered usually crawls or jumps on to the cloth lining without delay, so that boxing is not a lengthy process. When the required number are covered, a piece of fine silk gauze (chiffon) is placed over the upturned lid of the box at the bottom of the pan. The box is quickly lifted, fitted over its own inverted lid and gently forced down so that it does not quite shut. Thread can then be tied round the crack between the shoulder of the box and the lid. The lid may be removed, leaving the fleas securely imprisoned in a box with a glass bottom and a gauze cover in place of, or in addition to the lid. The process is somewhat easier if a loose fitting lid is kept specially for the process of fitting the gauze.

A more rapid method of boxing, useful for example in egg-laying experiments, is to first collect the required number of fleas in a tube and then to place in the pan a box prepared with cloth lining as described above. The lid of the box is slightly lifted and the

fleas are shot in by a rapid tilt of the tube and the lid closed immediately. With most fleas a little practice makes one deft enough to perform the operation so that few, if any, have time to escape. In the case of *P. irritans*, however, this frequently happens.

The method of storing in gauze-covered boxes described above as specially suitable in case of *P. irritans* is applicable for dealing with any species of flea that it is desired to test in regard to its aptitude to feed on man. It can also be adopted for feeding tests on other mammals, provided that their fur is closely cropped on the area of application. Feeding fleas from boxes is not without its advantage in other ways than that of convenience, for there is no danger of their escape or destruction by the host while feeding, a point of considerable importance if the number of specimens is small.

It has not been found possible to feed captive fleas on either the domestic cat or dog with the time at our disposal for this purpose. The dog was willing, but too anxious to play, while the cats were indignantly and successfully hostile.

With a tame rat the matter was easy and it was found quite unnecessary to secure the rat. By holding it beneath the left hand, head to wrist, with the two forefingers along its back and the box containing the fleas held with the right hand against the clipped patch above the tail, it was possible to give undisturbed feeds of from five to ten minutes and repeat as desired.

To test the length of life of fleas unfed under conditions as favourable as possible, the adults are put into small paper tubes, made of filter paper rolled round a penholder and secured with thread tied round them. The ends of these tubes are carefully blocked with plugs of cotton wool wrapped in fine gauze; the gauze is necessary to prevent the fleas from being lost in the plug. The tubes are buried in the sand pots previously described and kept in the places where the experiment is to be made.

A second method is simply to place the fleas in a small card jar with sand at the bottom; the fleas burrow in the sand and some species appear able to survive longer under these conditions than in tubes, for example, one specimen of *C. fasciatus* lived 95 days and one of *C. gallinae* 127 days. *X. cheopis* is also favoured by this method, but to *Ct. canis* and *P. irritans* it is of no advantage.

SECTION IV. GENERAL OBSERVATIONS ON BIONOMICS OF FLEAS.

1. EGGS (Plates XXX and XXXI). As might be expected, the general effect of cold or cool conditions is to slow down or prevent egg laying. In case of both *P. irritans* and *C. fasciatus* however it is possible to induce them to lay at any time by changing them in a warm moist atmosphere (about 75° F., humidity about 70). While *P. irritans* is the more responsive of the two, in neither case do the number of eggs laid under these conditions reach the summer average.

With *X. cheopis* the response to incubator treatment during the winter months is but slight; very few ova are obtained by removing females from the cages and placing them in an incubator at 75° F. for 24 hours. It appears necessary for egg production by this species that their host should be living in a warm atmosphere.

Ct. canis certainly lays under suitable conditions apart from season, as I have taken larvae from a dog's bed, indoors, as late as December.

C. gallinae is possibly more fixed in seasonable habit than the other species as regards egg laying. I have fed a number during the autumn months on myself, but, although kept constantly at a temperature of 75° F. except when feeding, no eggs were laid until spring.

2. LARVAE (Plates XXXI and XXXII). Rösel von Rosenhof reared fleas from the egg more than 150 years ago, and found that the larvae did not, as some supposed, feed on rotten wood. He discovered that they would feed on the bodies of their dead parents and could be reared on dead flies. The imminent failure of this source of diet with the oncoming of winter caused him to seek for another food. He made trial of the blood of some pet doves and proved that the larvae could be successfully reared on this food. No doubt—as pointed out in Report XXIX. Section 1. *Journal of Hygiene*, Vol. VIII. No. 2, May 1908, pp. 236 and 237—fleas may be reared on many kinds of organic matter if in a state of sufficiently fine division and fairly dry. The experience gained in the course of the present work, however, suggests that Rösel was very near to giving them their correct food and that the mainstay of the larval diet is the faeces of the adult fleas.

It may be that the necessity for this diet in case of larvae of a particular species depends upon the closeness of the association between the parent and the host fed upon. Fleas being chiefly, if not exclusively, nest or lair parasites, it is not surprising to find that the larvae should utilise as food the rich store of organic matter in suitable condition for assimilation that is afforded by the droppings of their parents.

It is an interesting speculation as to how far the adult's habit of wasteful feeding is the direct outcome of selective action, making a special provision for the larval food supply¹. The habit of sucking to such excess that some of the intake has to be voided is not restricted to fleas; instances are recorded of species of the Hymenoptera and Lepidoptera acting in the same way.

The other conditions provided by the nest or lair of the host in nature are also ideal for the flea larvae, or, to put it in other words, the larval requirements are adjusted to those that are most likely to obtain where the eggs are dropped. The necessary conditions of warmth and humidity are provided by the host's body, while the provision of bedding and careful choice of a dry situation all fit in with the needs of the larval stage of the parasite. When the host leaves its nest or lair the temperature and humidity fall together, but, so far as observation goes, a fall in temperature will only have the effect of slowing development; a low humidity, however, if prolonged, will be fatal even when accompanied by low temperature.

P. irritans would appear to have diverged from the other nest-breeding fleas in respect of the sensitiveness of its larvae to external conditions; possibly the progressive civilisation of its host has forced it to become more adaptable. Larvae of this species were successfully reared under circumstances that proved fatal to *C. fasciatus* (see Tables XVI and XXI), and, for example, were able to feed satisfactorily on crushed rat faeces when the larvae of the latter species failed. Probably the trend of selective action has been in the direction of producing a race of *P. irritans* able to feed on any possible rubbish in out-of-the-way corners. Undisturbed breeding places in such immediate vicinity to its host as to receive any appreciable quantity of the parental faeces would become gradually rarer as cleanliness and comfort succeeded to the crowding and filth of primitive conditions.

Experiments carried out with *P. irritans*, *X. cheopis*, *C. gallinae* and *Ct. canis* show that there are three larval instars, two of the three moults taking place while the larvae are in the active stage—the third skin being cast within the cocoon during the metamorphosis to the pupal condition. So far the number of individuals of each species

¹ It is very noticeable to what an extent sick animals are attacked by fleas; after death their fur may be found full of dried faeces of these parasites, as though the fleas not only had an instinct, like that of Dugald Dalgetty in Scott's *Legend of Montrose*, for a full meal—not knowing when they might get their next—but were desirous of making sure of the future of their species by bequeathing a rich store of food for their larvae.

followed up is small, and it is not possible to say if any variation in the number of larval instars occurs. It seemed quite possible that the very small males of *X. cheopis*, which are not infrequent, might have one instar less, but no evidence of this has been forthcoming.

The heads of several larvae in each stage were measured and gave the following approximate ratios :

<i>Ct. canis</i>	1st	·16 mm.	<i>C. gallinae</i>	1st	·16 mm.
	2nd	·18 mm.		2nd	—
	3rd	·22 mm.		3rd	·28 mm.
<i>X. cheopis</i>	1st	·14 mm.	<i>P. irritans</i>	1st	·18 mm.
		·16 mm.		2nd	·24 mm.
		·20 mm.		3rd	·28 mm.

When reared in cages, the situation chosen by the larvae for spinning varies considerably between *C. fasciatus* and *X. cheopis*. Those of *X. cheopis* may be found like those of *L. musculi* spun on fragments of cloth, etc. given to the host for bedding. With *C. fasciatus* the cocoons are chiefly in the sand or at the bottom of the cages, seldom or never on the cloth.

3. COCOONS (Plates XXXIII and XXXIV). The cocoon is an oval envelope of silk (in the case of *C. fasciatus* which may often be more aptly described as of cement), spun by the larva, its exterior being covered with sand, dust, or any small fragments of dry material that are available. These fragments are not worked into the fabric, and it is probable that the larva collects them and attaches them together with a few silk threads and then proceeds to thicken the interior of this loose case with further accretions of gummy silk from the inside. Not infrequently the cocoon will be spun against the side or bottom of the rearing receptacle, the larger particles of sand, etc. being utilised for choice.

The cocoon of *C. fasciatus* varies from a flimsy structure composed of a few silk threads holding together grains of sand or other material, after the fashion of a string bag, to a hard, strong case, in the manufacture of which a much greater quantity of silk must be used. This forms a dense, firm lining of a yellow or brownish colour to the cocoon, which in extreme cases is almost like thin horn. Cocoons of *C. fasciatus* are easier to open than those of the other species dealt with, owing to the brittle character of the silk used.

P. irritans spins a quantity of soft white silk when well fed, and its normal cocoon is tough and soft.

The cocoons of *X. cheopis* are tougher and softer than those of *C. fasciatus*, and have more in common with *P. irritans* in the matter of shape as well as of texture.

Cocoons of *Ct. canis* are in appearance and texture nearer to those of *P. irritans* than to those of *C. fasciatus*. Those of *C. gallinae* have little resemblance outwardly to the cocoons already mentioned, being for the most part of pure silk, strong, soft and dense, pale brown in tint, and just transparent enough to allow of a decision as to their being empty or occupied. They are fastened to and spun amongst the material of which the nest is formed although the sand, etc. is not so firmly adherent to the fabric of the cocoon as in the case of the other species reared. The larvae of *C. gallinae* will, however, in sand produce cocoons very similar in appearance to those of *C. fasciatus* although not so hard.

Within these cocoons the larva lies doubled into a loop with some space to spare, although the acute bend in the body suggests discomfort; after pupation there is considerable space unoccupied. There is some doubt whether in the different species the extensive periods of rest which occur within the cocoon are larval or imaginal. The pupal period appears to be comparatively short, not more than a few days, so far as it has been possible to ascertain, and it seems probable that, once the pupal state is entered into, it must be carried to a conclusion without interruption. It was at first thought that the removal of pupae from their cocoons would be necessarily fatal, but this does not seem to be the case¹. Resting larvae of *C. fasciatus*, as well as pupae of this and other species, have been taken from their cocoons and subsequently produced normal imagines. It is, however, likely that the normal course of development may be thwarted and a tendency to further rest be curtailed by removal, and it is improbable that such disturbed larvae could resist dry or otherwise unfavourable conditions. Certainly no instances of any continued delay in pupation have occurred in case of artificially opened cocoons.

RESTING COCOONS OF *C. fasciatus*. There seems good reason to think that the hard, strong types of cocoon commonly made by larvae of *C. fasciatus* are associated with the aestivating—hibernating or resting—habit: all grades, including the extreme, may be present in the same batch reared under precisely similar conditions. In some batches the resting tendency was found to be noticeably stronger than in others (Note November 1911, in the monthly cocoon series, *C. fasciatus* (Table

¹ In a few instances naked pupae have been found in the cages; probably the larvae had been disturbed whilst spinning.

XXXIII)). While the evidence is not conclusive that these hard cocoons are always associated with lying over, and the frail ones with prompt emergence, still in a majority of cases this would seem to be the position of affairs (see Experiment with Hard and Soft Cocoons). It is easy to understand how desiccation may be long withstood in the impervious horn-like envelopes. The chances of injury and attack, either by parasites or predatory insects, is certainly lessened by the strength of the cocoons, although it is not easy to see that even the more flimsy cocoons are in much danger from parasites penetrating.

4. ADULTS (Plates XXVII–XXIX and XXXI). EMERGENCE FROM COCOONS. All the species dealt with so far have the habit of clinging to a cocoon after emergence and the flea may be passed over as it will sometimes remain quiescent, even after shaking the tube or box in which the cocoons are kept. On the other hand, any disturbance, however slight may be followed by emergence. It is also a fact that, when cocoons are opened for examination, they are found to contain living fleas far too often for it to be reasonably supposed that they are all chance occasions in which opening has happened to coincide with emergence. This supports the conclusion that the fleas are normally in the habit of resting, at any rate for a short period and possibly for many days, before emergence, unless some disturbance gives warning of the approach of a possible host.

P. irritans has the habit of shamming death, by lying on its side with the legs drawn up close to its body. It was at first thought that this habit was confined to the human flea, but continued observation has proved that the other species dealt with share the habit, with the exception of *X. cheopis* only, in which it has not yet been observed. No doubt, as in the case of other insects, the sudden change from rapid movement to complete immobility is most puzzling to a pursuer and a most effective method of escaping danger.

DANGER FROM HOST. Observation suggests that rats, dogs and cats frequently take considerable pains to rid themselves of fleas, especially on their first assault. It is noteworthy that rat fleas do not attack indiscriminately, that is to say, they pick out special points of vantage, *X. cheopis* making for the shoulders, neck and chest or for a spot beneath the forelegs¹. *C. fasciatus* has a favourite spot just above the

¹ It will be remembered that in Part III of the Report published in the *Journal of Hygiene*, Vol. VI, No. 4, Sept. 1906, pp. 465–6, this preference of *X. cheopis* for the cervical region was noted.

tail. A number of *C. gallinae*, however, did not show any discrimination in their attacks on a rat.

PERIODIC BREEDING. The results of cocoon experiments, see p. 534, both as regard *P. irritans* and *C. fasciatus*, afford evidence of the existence of a seasonal habit as regards the emergence of both species; and in case of *Ct. canis*, *C. gallinae*, *L. musculi* and our English stock of *X. cheopis*, the evidence is even stronger. With *Ct. canis* and *C. gallinae*, however, the habit is probably in the process of modification in the direction of continuous reproduction in response to the domesticated character of its hosts.

In nearly all insects seasonal habits are strongly ingrained, owing to the direct and powerful effects of climatic conditions on their constitution; with parasitic insects such as fleas a double adjustment is necessary, firstly in response to direct conditions, secondly in relation to the effects of seasonal changes on their hosts' habits. This latter factor must be most important in moulding flea bionomics, as the habits of flea hosts vary from a continual occupancy of lairs by some wild animals, such as bats and carnivora, to a spasmodic appropriation of convenient situations by wandering animals, such as rats. In the case of birds there is a strictly limited seasonal occupation of the old nest for two or three months only. It is also probable that, even when the home of the host is of a permanent character, and more or less in continual occupation, flea increase will be adjusted to the breeding periods of its host; it is unquestionably at these times that the best opportunity occurs for uninterrupted feeding on the part of the adult fleas, and a consequent ample food supply for their larvae.

Nomadic animals which do not possess the homing instinct are far less likely to have specific fleas allotted to them than other animals. With *P. irritans* there seems little need for any adjustment in temperate climes. With *C. fasciatus* the conditions have also become modified, but the primitive habits of this species are less likely to have suffered change than those of *P. irritans*, as only a portion of the rat population is closely enough associated with dwellings for their parasites to enjoy the full benefits of domestic life.

So far as ascertained in the course of these experiments, *C. gallinae* has a very definite breeding season during spring and early summer—the adults emerging in the spring and living through winter. Its habits are loose in other respects and it appears to have so adjusted itself as to be able to obtain sufficient nourishment for breeding purposes from the blood of its host almost as quickly as *P. irritans*. It is possible

that the exigencies of an existence begun in an *empty* nest have forced upon it the necessity of leaving no chance untried of providing for the future of its race.

BREEDING WHEN FED ON AN ALIEN HOST. In view of the fact that the species experimented with, can live for considerable periods when fed on the blood of an alien host the question of their breeding under these circumstances becomes one of considerable interest. Pairing certainly took place but no eggs were developed, so far as could be observed without dissecting specimens, certainly none were laid. This was at first thought to be due to the nature of the food alone, but it subsequently appeared to be connected with the conditions of captivity and the comparatively limited opportunities to feed.

C. gallinae. Among some fleas taken from a dog in November 1910, was a female specimen of *C. gallinae*; this flea was fed on a human host throughout the winter and during March laid a few ova which proved to be infertile. During February 1911 seven specimens of *C. gallinae* were taken from a deserted Tit's nest and were fed on a human host. Copulation was seen to occur among them but most of the males soon died off, the female specimens living longer, and one which survived for 41 days laid some 20 eggs during March. These eggs proved to be fertile, and larvae to the number of 16 were reared without loss, a few being used as specimens, the remainder producing adult fleas.

C. fasciatus. Trials lasting from 4 to 79 days (average 27), were carried out with three batches of fleas, comprising 22 individuals, but no ova were laid. A further experiment with this species, consisting of a batch of 16 fleas boxed with a tuft of rat's hair, was in progress for over a month, but no eggs resulted, although the box was kept in the humid incubator at 75° F. and the fleas were seen to copulate on several occasions. Both *C. gallinae* and *C. fasciatus* will pair before feeding, in fact shortly after emergence.

X. cheopis. Two batches, consisting of 19 and 15 individuals respectively, lived on a human host for from 18 to 104 days (average 63 days), but no eggs were laid.

P. irritans. Two attempts to breed this species when fed on rats failed to produce either eggs or brood. In one case six specimens were put into a cage with a young rat, and one individual lived for 40 days, but no larvae could be found nor did the cage produce any fleas. In a second attempt *P. irritans* was fed on the rat by the box method; the length of life was, however, very short in this case, none of the fleas

surviving more than 14 days, and it seems probable that little if any actual feeding was done. No eggs were laid. It is possible that *P. irritans* may breed to a limited extent on the dog as we occasionally rear a few individuals from among the abundant supply of flea larvae in the dog's bed, but it is probable that the ova which give rise to these larvae were laid within a day or so after the fleas were transferred to the dog's coat.

Ct. canis. Three batches consisting of 14 individuals, three of which were captured and the remainder bred, were fed on a human host for varying periods of from 15 to 245 days (average 71 days), but no ova were laid. A fourth batch of 20 specimens—fresh specimens being added from time to time—were boxed with a quantity of the dog's hairs placed in the box, to see if the association had any effect and fed for some months. No eggs were laid, however, although the box was kept in the humid incubator at 75° F. except during feeding times. In this case it can hardly be a question of season, for the same species was breeding successfully in the dog's bed while the experiment was in progress; this was shown by the presence of eggs.

About 60 freshly emerged specimens of *Ct. canis* were put into a cage with a young rat, none of them survived a month and no brood was discoverable, nor did any fleas emerge later in the cage. Ten specimens fed on the rat by the box method lived for varying periods of from 24 to 58 days (average 41 days), but no ova resulted.

Ct. felis. Only one attempt has been made with this species; five individuals comprising both sexes were fed on man for periods varying from 139 to 185 days. No eggs were laid.

Ova were successfully obtained from boxed specimens of *Ct. canis* taken from the dog, and of course ova of both *C. fasciatus* and *X. cheopis*, are freely obtained by the box method when the adult fleas are taken from the rat cages. This tends to suggest that some specific character of the blood ingested is the controlling factor in egg production; it must, however, be noted that when either *C. fasciatus* or *X. cheopis* has been fed by means of the box method on the rat no eggs have resulted. Three attempts were made with *C. fasciatus* fed on a rat by the box method; 24 individuals were used and survived from four to 106 days (average 23 days), yet no eggs were laid. A later attempt with rat's hair in the box has also proved negative; in this case 20 fleas were fed on a rat for some three weeks.

In comparison with *P. irritans*, all the other species observed are in much closer association with their hosts. They probably are

accustomed to feed in a much more leisurely manner and at more frequent intervals than the human flea. It was therefore decided to test the effect of more frequent feeding on man, and batches of *X. cheopis*, *C. fasciatus* and *Ct. canis* were fed twice instead of once a day, the second feeding being of longer duration than the usual 15 minutes.

The experiment proved partially successful; *C. fasciatus* was found to lay eggs freely and a gradually increasing stock of this species has been maintained, apart from any other source of supply. *X. cheopis* laid sparingly, but although fleas were reared from the resulting larvae and added to the adult feeding box, the experiment could not have been continued were it not that individuals reared on the rat were also added to keep up the supply of parents. *C. gallinae* proved a somewhat better layer than *X. cheopis* but stocks could not be long maintained without the addition of adult fleas from other sources.

It is of course quite improbable that *C. fasciatus* could perpetuate its race by adopting a human host, as no proper provision would be available for the larvae. *X. cheopis*, however, is more adaptable in the matter of food and this difficulty would not apply.

No success has, however, attended any attempts with *Ct. canis*. The adults of this species, like those of *X. cheopis*, practically live upon their host, and possibly practise very constant feeding. It may be that the two opportunities per day on an alien host are not sufficient and that very frequent feeding might induce *Ct. canis* to lay eggs when fed on man¹.

The whole of the foregoing evidence is entirely opposed to the view that fleas can continue breeding in the absence of food for the adults.

To account for the records of large numbers of fleas existing in long deserted dwellings, or the unoccupied beds of animals, the explanation

¹ Later trials with this species have proved quite successful. When given opportunities of feeding on a human host, which extended to five hours daily, a considerable number of eggs were laid, all of which, however, failed to hatch. The possible feeding period was then increased to at least twelve hours per day and eggs were freely laid, a large proportion of which hatched. The resulting larvae were successfully reared and over thirty adult *Ct. canis* emerged from the cocoons spun by these larvae. There seems no obvious reason why dog fleas should require so much longer for feeding than those associated with rats or fowls, but I am inclined to think that the solution of the problem is in some way bound up with the needs of the larvae in the matter of food. The increased number of eggs laid by the twice fed *P. irritans* (see Sect. V., Influence of Food Supply on Fertility) appears to me to favour such a suggestion.

has been put forward that in the presence of ample nourishment for the larvae, generations of fleas might be reared in spite of an entire absence of any food supply for the adult fleas. It is assumed that the nutriment absorbed during larval existence suffices for egg production, as well as for the development of sperms in fleas; this is of course the case with many insects.

As stated elsewhere in this report, copulation certainly takes place among newly emerged unfed fleas. The habit has been commonly observed in most of the species dealt with and may be universal. Dissection of the larvae, pupae and imagines proves that developing ovaries may be found in the full fed and resting larvae, and onwards through the pupal period in various stages of development. The ova, however, are undersized, even after active imaginal existence has started. Throughout the whole course of these experiments oviposition has always been preceded by several days' feeding, and no single instance was observed in which an egg was laid by an unfed female; all the evidence points to the necessity of feeding the adult for the full development of eggs.

The fertility experiment conducted with adults of *P. irritans* to ascertain if length of feeding time had any result on the percentage of eggs hatching also supports this view; it was found that feeding twice a day resulted in a much larger number of eggs than a single daily meal. The number of opportunities for feeding had, however, no noticeable influence on the proportion of eggs which hatched.

When feeding the different species on man by the box method, a considerable difference was noticed in the character of the faeces deposited on the gauze and sides of the box. *P. irritans*, as was to be expected, fed to the greatest excess, blotching and splashing the sides of the box and gauze cover with semi-fluid dejecta, which were frequently bright red in colour. *Ct. canis* tends to deposit its faeces in little piles one upon the other, suggesting that the dejecta in this case were of a more pasty nature than those of the human flea. *C. gallinae* makes a deposit of neat raised dots, very similar to that of *C. fuscatus*, but the feeding of the latter species, to judge by the deposit, seems to be of a less vigorous character. The faeces of the other species tend to be darker in colour than those of *P. irritans* and I have never seen any trace of the fresh blood appearance in the former. It seems not improbable that this difference is due, not so much to a difference in digestion, as to the speed of feeding. In a few minutes *P. irritans* reaches the stage when it voids old accumulations from the gut, whereas,

in the case of the other species, the feeding period expires ere they have been able to obtain anything like a surfeit.

IRRITATION CAUSED BY FLEA BITES. The irritation caused by fleas on the human body is known to vary very widely in case of different persons. My assistant developed a distinct papular rash on his arm after feeding some *C. fasciatus* thereon, although the attacks of *P. irritans* did not in any way incommode him. Personally, the bites of fleas do not cause me any irritation whatever, although I am keenly conscious of their progress over my skin. I continued to feed upon myself the particular batch of *C. fasciatus* which caused the trouble referred to above, until they died some weeks later, but without any signs of a rash appearing. Since then I have fed numbers of individuals of the various species upon the same skin area for months together without inconvenience, and my assistant has also fed many specimens including *C. fasciatus*, for several separate periods of a week or more, while I was absent from home, without any recurrence of the rash.

A similar instance of a rash caused by *C. fasciatus* is mentioned by Chick and Martin (1911) and the question of irritation caused by rat fleas and the reaction to them is discussed briefly by Boycott (1912).

LENGTH OF LIFE. The great range of individual variability presented in the earlier stages of the fleas' existence is maintained as regards the length of the adults' life; of this the experiments give abundant examples. Some individuals die off quite early in the experiment, especially when the host is alien. The majority go steadily on for a time, dropping out gradually in a middle period. A few become thoroughly adjusted to their environment and food and far exceed all their companions in longevity. These very long lived specimens are always, in my experience, females.

SECTION V. EXPERIMENTAL STUDY OF THE INFLUENCE OF EXTERNAL CONDITIONS UPON THE VARIOUS STAGES IN THE LIFE HISTORY.

It will be noticed that in the earlier experiments there is fair continuity between the tables dealing with ova and those concerning the larval period (see ova Tables I, II, III, and V; larvae Tables XVI, XVII, XVIII, XXI, and XXII, up to March 1911). The cases where experiments dealing with ova were not continued through the larval stage are few and the deficiency was owing to the young larvae being required for bacteriological experiments. There are also cases in which larval experiments have no previous record. This is due to the difficulty

and great labour involved in determining the exact time and circumstances of the hatching. There is no artificial break in continuity from larval to cocoon stage in this series, but many of the larval records end before the spinning period is reached (see Tables XVI, XX and XXI).

In the experiments subsequent to March 1911, no continuity is attempted from the egg- through the larval- to the cocoon stage, all three are treated separately. The hatching experiments were concluded at the emergence of the young larvae, which were then returned to cages in which reserve stocks were kept. For the rearing experiments only those larvae were used which hatched from eggs laid and kept at 75° F.; they were removed daily and divided into batches for distribution among jars maintained under different experimental conditions. For tests with cocoons, full sized larvae were taken from the breeding cages (or breeding jars in case of *P. irritans*) and placed with food and sand at 75° F. The cocoons were then collected daily in batches and distributed to the experimental jars as in the case of larvae.

1. EGGS.

In the experiments made with ova before the end of January 1911, the method adopted was to place the eggs on paper or cloth in the receptacle in which the larvae were to be reared, the sand and food being added before the eggs were put in. This plan was adopted so as to give the larvae every chance of survival, but it entailed the rather cumbersome process of examining the ova individually to see if they had hatched; the periodic examination of the eggs was also rendered difficult because they were frequently dislodged or dragged off the paper or cloth into the sand, presumably by the larvae in their attempts to get clear of the shell¹.

After the first experiment with newly hatched larvae had revealed their fasting powers the much more convenient plan was adopted of placing each batch of eggs in a clean dry tube and recording the number of larvae removed daily to the receptacle in which they were to be reared. A quantitative examination of the empty shells was also made in a number of cases and the results were found to be in close agreement with the record of larvae transferred. The earlier

¹ The egg shell is ruptured, not eaten through. A long slit on the side of the egg is made through which the larva escapes. This slit appears to give the empty shells very elastic or spring-like qualities, for they will frequently fly at the slightest touch of a camel hair brush.

method may therefore be taken to give correct results of hatching save that the number of eggs remaining on the cloth, etc. was often considerably less than the actual number of ova used and the percentage of fertility is apt to be lowered owing to the fact that fertile eggs are more apt to be dislodged than the infertile.

C. fasciatus. A comparison of moistened with unmoistened conditions (Tables I and II) in the incubators shows, in case of *C. fasciatus*, that moistened conditions are on the whole favourable to hatching of eggs while a slight tendency in the opposite direction is shown in case of incubator 75 Wet. The advantage of humidity is also seen by comparing results of experiments in the incubators in which hatching took place in tubes with those where jars or boxes were used. The proportion of eggs is higher in the former case presumably because the conditions were much more favourable to the retention of moisture. In the experiment made in Incubator 75 Wet the result is, however, doubtful and, in case of hatching in the cellar and laboratory cupboard, the reverse is true. The favourable effect of humid conditions in the general experiments is, as a whole, not very apparent as it is to a considerable extent masked by the wide range of individual variation which appears to apply in some measure to the fertility of eggs as well as the other characters of these insects. When, however, we select a single experiment planned on lines to bring out this point the advantage of humid conditions seems unmistakable.

Experiments with ova of C. fasciatus in card jars (unmoistened)
Table II.

Date	Incubator	Temperature	Average humidity during progress of experiment	Number hatching
21 Dec. 1910	75 Wet	74.2 F.	.75	28 out of 32 = 87 $\frac{1}{2}$ %
21 Dec. 1910	75 Dry	75.7 F.	.46	14 out of 26 = 54 $\frac{1}{2}$ %
24 Dec. 1910	85 Wet	83.6 F.	.70	16 out of 26 = 62 $\frac{1}{2}$ %
24 Dec. 1910	85 Dry	83.8 F.	.59	9 out of 28 = 32 $\frac{1}{2}$ %

To test the matter further, a series of experiments, dealing with eggs alone, was planned to detect, if possible, the relative effect of temperature and humidity on the number of eggs laid, their fertility and the percentage hatching (see Table IV). A large number of *C. fasciatus* were taken from the cages and put together ("bulked"), they were then counted out into batches, each batch containing 50 to 70 females. The males were put in with the females, but not counted—there would be, however, approximately the same proportion of males

in each batch. Each batch was placed in a 1 $\frac{1}{4}$ " glass-bottomed box with a strip of cloth arranged to receive the eggs as described—the box being then buried in a jar of sand and placed in incubator, cupboard, cellar, etc. In series I the ova were allowed to hatch in the place in which they had been laid; in series II eight separate boxes containing eggs laid in different situations were all placed to hatch out in incubator 75 Wet, while in series III the experiment was varied by putting the boxes containing the fleas into incubator 75 Wet, bulking the ova laid, and then putting them away in batches of 50 to hatch out in different places (see Table IV). The four batches which were in incubator 75 Wet throughout (first experiment in each series), should give a measure of variability, and it will be seen that this is a wide one, viz. in the four experiments the proportion of eggs hatched varied from 71 to 85 %. The difference between the incubators 85 Dry and 75 Dry, and 85 Wet and 75 Wet, is not so marked as in the contrasted experiment referred to above under date of December 1910; this is most probably attributable to the fact that the humidity of incubator 85 Dry and 75 Dry, though still considerably lower than in 85 Wet and 75 Wet, was higher in July 1911 than in December 1910; being, for 85 Dry, .66 in July 1911, as against .59 in December 1910, and for 75 Dry, .55 as against .46. Although the effects of temperature and humidity are clearly apparent, apart from individual variability, some of the finer points that it was hoped to throw light upon are more or less submerged, or reduced to the value of possible tendencies. The results of the three series of experiments set out in Table IV may be summarized as follows, using incubator 75 Wet as a standard:

The conditions in 85 Wet are detrimental to hatching but favourable to the fertility of the eggs laid.

The conditions in 75 Dry are detrimental to hatching but favour the fertility of the eggs laid.

The conditions in 85 Dry are detrimental to hatching and perhaps to the fertility of the eggs laid as well.

The conditions in cellar are favourable to hatching but detrimental to the fertility of the eggs laid.

The conditions in warm cupboard are detrimental to hatching but favour the fertility of the eggs laid.

The conditions in laboratory cupboard are detrimental to hatching but favour the fertility of the eggs laid.

The conditions in beehive are detrimental to hatching but favour the fertility of the eggs laid.

With regard to the suggestion above, that low humidity is favourable to the fertility of the eggs laid under these conditions it must be

admitted that the facts are equally well explained on the assumption that the fertility is in inverse ratio to the number of eggs laid. High temperature when combined with a fair to high humidity is especially favourable to egg laying. It will be observed that in incubators 85 Dry and 85 Wet, which were on the whole not favourable to the fertility of the eggs laid in them, the number of eggs laid per female was usually greater than in those places which appear more favourable to fertility.

P. irritans. A similar experiment was planned (Table VI) on lines as nearly as possible to the foregoing one with *C. fasciatus*.

20 females, after feeding for one week, were divided into four batches and placed in boxes with a number of males which had been treated similarly. The batches A, B, C and D were fed daily for 15 to 20 minutes and kept for the rest of the day in sand pots placed in incubators 75 Wet, 75 Dry, 85 Dry, and the cellar respectively; eggs were removed from the boxes every three or four days. The females of each batch A, B, C and D were unchanged throughout and those that died were not replaced. The males, however, were replaced as they died and their numbers were increased from three to 10 in each box, but this increase was always made at the same time from a single batch that had emerged within a few days of each other, so as to preserve similar conditions for each batch, the object being to vary temperature and humidity only.

After the batches had been kept under the original set of conditions for some two or three weeks they were submitted to fresh ones and after a further period returned to their original situations. In the first two experiments of each batch the ova were allowed to hatch in the situation in which they had been laid, later they were all put into incubator 75 Wet to hatch in order that the effect of varied conditions on their fertility might be tested. Batch A started in incubator 75 Wet, was transferred to 85 Dry and returned to 75 Wet. Batch B started in incubator 75 Dry, was transferred to the cellar and returned to 75 Dry. Batch C started in incubator 85 Dry, was transferred to 75 Wet and returned to 85 Dry. Batch D started in the cellar, was transferred to incubator 75 Dry and returned to the cellar. Finally (see Table VI (a)) all the different batches of fleas A, B, C and D were kept under similar conditions, the eggs laid were "bulked," divided into batches and put away in different places to hatch.

The experiment shows that hot dry conditions were most favourable to oviposition, the results being as follows:—the adults in incubator 85 Dry (temperature 83·9° F., humidity ·61) showed 1·54 eggs per

female per day: incubator 75 Dry (temperature 74·6° F., humidity ·60) 1·40: incubator 75 Wet (temperature 75° F., humidity ·77) 1·11 and cellar (temperature 63° F. max., 62° F. min., humidity ·94) ·70 eggs per female per day.

The effect of transference from the coolest conditions to a higher temperature and drier atmosphere (batch D) was to increase egg laying, and for reverse conditions (batch B) to decrease it—both changes having a progressive effect; that is to say, in both cases the increase or decrease was more marked in the second period of time than the first. On the other hand, change from the moderate conditions of heat and moisture in incubator 75 Wet (batch A) to the hotter and drier incubator, 85 Dry, tended at first to check but subsequently to increase egg laying, while the reverse change (batch C) caused a progressive rise beyond an already high percentage. The return of the boxes to their original situations produced a rise with batch A, a reduction with batch C, a marked increase with batch B and an equally noticeable drop for batch D.

The final transfer of the batches to incubator 75 Wet produced an increase in the number of eggs laid in case of batches B and C, presumably in response to the more humid conditions; a marked rise in batch D was also to be expected. In case of batch A which had remained in incubator 75 Wet for two successive periods the number fell in the second. This may have been an oscillation in the reverse direction after its spurt following upon the previous change, or, more probably, in response to the falling humidity in incubator 75 Wet itself.

Individual variation is not so apparent in this experiment as it was in that dealing with *C. fasciatus*. This was to be expected in view of the fact that we are here dealing with only 20 females of *P. irritans*, whereas with *C. fasciatus* hundreds were taken from the cages on several different occasions.

The influence of temperature and humidity upon fertility, apart from the percentage hatching, is shown by a comparison of the percentage of eggs hatching in incubator 75 Wet after being laid under other conditions and may be studied from Table VI (*b*) of which the details have been collected from various experiments in Table VI. For instance, the eggs of batch B when laid in incubator 75 Dry show a higher percentage hatching in incubator 75 Wet (58%, Table VI (*b*)) than in incubator 75 Dry (44%, Table VI); when the ova of this batch were laid in incubator 75 Wet and placed in incubator 75 Dry to hatch, the proportion hatching was lower still. In these figures, other things being equal, we have evidence that the conditions in incubator

75 Dry are favourable for fertility of the eggs laid there, as shown by the increased proportion hatching when moved to suitable conditions for this second stage.

In Table VI (a) are given the results of a special experiment to determine the best conditions for hatching, all the eggs used being laid in incubator 75 Wet and placed in different places to hatch out. From the results it seems clear that the conditions of heat and drought in incubator 93 Dry are detrimental to hatching¹, and this is also the case in incubator 75 Dry, which is still drier but not so hot; the cellar, on the other hand, while detrimental to fertility (see Table VI (b)) is favourable to hatching.

The experiments with *P. irritans* do not lend any support to the suggestion mentioned in case of *C. fasciatus*, viz. that fertility might bear some relation to egg production, being in inverse ratio to the number of eggs laid. That is to say that, under the forcing conditions of high temperature, immature eggs are deposited. With *P. irritans* it would rather seem that the high temperature induced not only free laying, but increased facility for sperms to come in contact with the eggs.

The suggestions to be gained from the above experiments as regards hatching and fertility of eggs are the following

P. irritans. Incubator 75 Wet as Standard.

The conditions in incubator 85 Dry favour fertility and are detrimental to hatching.

The conditions in incubator 75 Dry favour fertility and are detrimental to hatching.

The conditions in the cellar are detrimental to fertility but favourable to hatching.

X. Cheopis. Considerable difficulty was experienced in getting sufficient stock to carry out egg experiments with this species on as full a scale as with *C. fasciatus*. The rapid breeding that took place during the summer of 1911 led to an expectation of a liberal supply in the autumn and winter, but experience proved that the numbers dwindled rapidly when the hot weather had passed and the effects of renewed breeding were not apparent in the cages until late spring and early summer of 1912. There is no doubt that this species requires an average temperature of from 65 F. to 70 F. for rapid increase, and, quite apart from the question of seasonal habit, the temperature in the laboratory, where the cages were kept, fell so low during the night as to check breeding except during really warm summer weather.

¹ The continuity of this experiment with the one detailed in Table VI has been somewhat impaired by a rise in the temperature of incubator 85 Dry from 85° F. to 93° F. in order to carry out the experiments dealing with *X. cheopis*.

The methods followed for the study of external conditions upon fertility and hatching of eggs were the same as those adopted with *C. fasciatus*, which have already been described; the results are set forth in Tables VII and VIII.

From a study of the earlier experiments (see Table VII) performed during July and November 1911 and January 1912, it will be noticed that either a seasonal change takes place with regard to the fertility of the ova (*i.e.* eggs are less fertile when laid out of season), or else the reduction in humidity, which is of general occurrence in the incubators during the autumn, has a detrimental effect on their hatching. Whatever be the cause it is patent that cool conditions, such as are presented by the laboratory cupboard, cellar and beehive during autumn, winter and spring, are fatal to *all* the eggs subjected to them. The falling off in the percentage of eggs hatching of both *P. irritans* and *C. fasciatus* that are placed at temperatures in the neighbourhood of 55 F. or lower is also very marked (see *C. fasciatus* Table IV, cellar 20th April 1911, and *P. irritans* Table VI), although there is no case of the complete failure of all the eggs in any one batch.

The results of the threefold test with *X. cheopis* (Table VIII) are disappointing in view of the small number of eggs deposited, and show to an even greater extent than *C. fasciatus* the variability as regards the percentage of eggs hatching in incubator 75 Wet on different dates. This difference must be taken into account, nor can we draw any safe conclusions as to the effects of the varied conditions to which the eggs were subjected, unless the trials took place on identical dates. Under these conditions, as the eggs used were all portions of a single batch or all laid by detachments of one body of females, the difference may be taken to represent the effect of the varied condition alone.

The results, summed up in a brief table of conclusions, as in the case of *P. irritans* and *C. fasciatus*, may be stated as follows:

Incubator 75 Wet as Standard.

The conditions in incubator 93 Wet are unfavourable but not necessarily fatal to both fertility and hatching.

The conditions in incubator 75 Dry are on the whole somewhat less favourable to both fertility and hatching than 75 Wet but any change from one to the other is distinctly unfavourable.

The conditions in incubator 93 Dry are certainly very unfavourable, probably fatal, to hatching, but as regards the fertility of the ova laid are not unfavourable, about *parallel* with 75 Dry.

The conditions in cellar are very detrimental to egg laying and unfavourable to hatching; the effect on fertility is questionable.

The conditions in warm cupboard are somewhat unfavourable for hatching but not to the fertility of eggs laid.

The conditions in laboratory cupboard are distinctly unfavourable for laying, hatching and perhaps fertility as well.

The conditions in beehive are distinctly unfavourable for laying, hatching and perhaps fertility as well.

The conditions in ice-chest are fatal.

The evidence for these conclusions is admittedly even less decisive than it was for the tabulated conclusions in regard to the other species. They must be viewed in the light of suggestions and in no way final.

TABLE I. *Ova C. fasciatus. Influence of temperature and moisture on the proportion hatching; all eggs laid in incubator "75 Wet."*

Date	Receptacle			Temperature	Moistened	Number examined	Number hatched	Percentage
	Tube	Box	Jar					
1 Dec. 1910	×			74·0	Water at start	11	11	100 0/0
6 Dec. 1910	×			74·1	Water at start	21	18	85 0/0
8 Dec. 1910	×			74·3	Water at start	14	14	100 0/0
*28 Dec. 1910			×	74·6	Water at intervals	29	21	73 0/0
13 Jan. 1911	×			74·7	Water at start	16	13	81 0/0
* 4 Mar. 1911			×	74·6	Water 1 c.c. daily	36	13	36 0/0
Total hatched	90 0/0		53 0/0					
*28 Dec. 1910			×	74·6	Urine at intervals	30	23	77 0/0
* 4 Mar. 1911			×	74·6	Urine 1 c.c. daily	36	25	69 0/0
Total hatched			73 0/0					
(b) Incubator 75 Dry, moistened.								
8 Dec. 1910	×			74·7	Water at start	19	12	63 0/0
17 Dec. 1910	×			76·2	Water at start	25	21	84 0/0
* 3 Jan. 1911			×	76·1	Water at intervals	21	13	62 0/0
*31 Jan. 1911			×	76·6	Water daily	27	20	74 0/0
28 Feb. 1911			×	76·0	Water 1 c.c. daily	30	17	57 0/0
Total hatched	75 0/0		64 0/0					
* 3 Jan. 1911			×	76·1	Urine at intervals	19	12	63 0/0
*31 Jan. 1911			×	76·6	Urine daily	39	21	54 0/0
28 Feb. 1911			×	76·0	Urine 1 c.c. daily	30	15	50 0/0
Total hatched			55 0/0					
(c) Incubator 85 Wet, moistened.								
1 Dec. 1910	×			83·5	Water at start	17	11	65 0/0
8 Dec. 1910	×			83·8	Water at start	8	7	88 0/0
*30 Dec. 1910			×	84·2	Water at intervals	20	16	80 0/0
*24 Feb. 1911			×	83·7	Water 1 c.c. daily	36	21	58 0/0
Total hatched	72 0/0		66 0/0					
*30 Dec. 1910			×	84·2	Urine at intervals	27	24	88 0/0
*24 Feb. 1911			×	83·7	Urine 1 c.c. daily	36	19	53 0/0
Total hatched			68 0/0					

* against experiments of the same date signifies that a single batch of eggs was divided.

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(d) Incubator 85 Dry, moistened.

Date	Receptacle			Temperature	Moistened	Number examined	Number hatched	Percentage
	Tube	Box	Jar					
*25 Nov. 1910			×	84.1	Water at intervals	15	8	53%
1 Dec. 1910	×			83.8	Water at start	18	11	61%
6 Dec. 1910	×			84.0	Water at start	20	18	90%
8 Dec. 1910	×			84.0	Water at start	17	11	65%
17 Dec. 1910	×			84.7	Water at start	24	19	79%
*30 Dec. 1910			×	83.6	Water at intervals	17	15	88%
*27 Jan. 1911			×	85.1	Water daily	16	9	56%
Total hatched	75%		66%					
*25 Nov. 1910			×	84.1	Urine at intervals	18	13	88%
*30 Dec. 1910			×	83.6	Urine at intervals	23	21	91%
*27 Jan. 1911			×	85.1	Urine daily	10	4	40%
Total hatched			75%					

(e) Warm Cupboard, moistened.

* 3 Feb. 1911			×	59.0	Water daily	25	19	76%
* 8 Mar. 1911			×	60.0	Water 1 c.c. daily	35	16	48%
Total hatched			58%					
* 3 Feb. 1911			×	59.0	Urine daily	19	12	62%
* 8 Mar. 1911			×	60.0	Urine 1 c.c. daily	35	12	34%
Total hatched			42%					

TABLE II. *Ova C. fasciatus. Influence of temperature and humidity on proportion of eggs hatching; all eggs laid in incubator "75 Wet."*

(a) Incubator 75 Wet, unmoistened.

Date	Receptacle			Temperature	Humidity	Number examined	Number hatched	Percentage
	Tube	Box	Jar					
5 Aug. 1910	×			75	.66	5	4	80%
11 Aug. 1910		×		75	.69	10	6	60%
24 Sept. 1910			×	74	.73	20	16	80%
30 Sept. 1910		×		74.1	.77	7	5	71%
16 Oct. 1910	×			74	.73	15	15	100%
19 Oct. 1910		×		74	.66	11	9	82%
30 Oct. 1910	×			73.7	.72	6	6	100%
						13	13	100%
21 Dec. 1910			×	74.2	.75	32	28	87%
13 Jan. 1911	×			74.7	.74	17	14	82%
14 Feb. 1911	×			74.5	.76	14	10	71%
14 Feb. 1911	×			74.5	.76	19	12	63%
14 Feb. 1911	×			74.5	.76	18	12	67%
Total hatched	80%	72%	85%					

* against experiments of the same date signifies that a single batch of eggs was divided.

(b) Cellar.

Date	Receptacle			Temperature		Humidity Approximate	Number examined	Number hatched	Percentage
	Tube	Box	Jar	Max.	Min.				
31 July 1910	×			59	58	·93	2	1	50 %
4 Aug. 1910	×			59	58	·93	5	4	80 %
14 Aug. 1910	×			62	60	·93	3	1	33 %
7 Oct. 1910			×	58	57	·93	22	19	86 %
22 Oct. 1910	×			56	55	·93	7	3	43 %
Total hatched	53 %		86 %						

(c) Incubator 85 Dry, unmoistened.

24 Dec. 1910		×		83·8		·59	28	9	32 %
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(d) Incubator 85 Wet.

30 July 1910	×			85		·75	7	4	57 %
4 Aug. 1910	×			85		·74	6	4	66 %
10 Aug. 1910		×		85		·73	11	1	9 %
9 Sept. 1910		×		85		·70	5	3	60 %
22 Sept. 1910			×	85		·72	20	12	60 %
13 Oct. 1910	×			84·5		·72	11	9	82 %
5 Nov. 1910	×			84·7		·72	15	14	93 %
24 Dec. 1910			×	83·6		·70	26	16	62 %
Total hatched	74 %	25 %	61 %						

(e) Incubator 75 Dry.

21 Dec. 1910		×		75·7		·46	26	14	54 %
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(f) Warm Cupboard.

				Max.	Min.	About			
6 Aug. 1910	×			69	67	·60	3	3	100 %
10 Oct. 1910			×	74	69	·60	16	10	61 %
22 Oct. 1910	×			73	69	·60	6	5	83 %

(g) Laboratory Cupboard, unmoistened.

				Of Laboratory Approximate only		Approximate			
25 July 1910	×			60		·84	3	3	100 %
6 Aug. 1910		×		60		·84	5	2	40 %
12 Aug. 1910		×		60		·84	6	4	66 %
18 Aug. 1910	×			60		·84	6	3	50 %
27 Sept. 1910			×	60		·84	9	6	66 %
16 Oct. 1910		×		60		·84	16	15	94 %
19 Oct. 1910		×		60		·84	8	5	63 %
Total hatched	66 %	80 %	66 %						

TABLE III. *Ova C. fasciatus*. Influence of varying the temperature and humidity upon the proportion of eggs of the same batch hatching; all eggs laid in incubator "75 Wet."

			Unmoistened.			No.	No.	Per-
Date	Receptacle	Place	Temperature		Humidity	exam.	hatched	centage
19 Oct.	Box	Incubator 75 Wet	74		66	11	9	82 %
		Lab. Cupboard	60 approx.		84 approx.	8	7	87 %
			Max.	Min.				
22 Oct.	Tube	Warm Cupboard	73	69	60	6	5	83 %
		Cellar	56	55	90	7	3	43 %
Moistened.								
1 Dec.	Tube	Incubator 85 Wet	83.5		Water at start	17	11	65 %
		" 85 Dry	83.8		" "	18	11	61 %
6 Dec.	Tube	" 75 Wet	74.1		" "	21	18	85 %
		" 85 Dry	84.0		" "	20	18	90 %
8 Dec.	Tube	" 75 Dry	74.7		" "	19	12	63 %
		" 85 Dry	84.0		" "	17	11	65 %

NOTE. Moistened by means of a disk of blotting-paper beneath the sand with a projecting tag.

TABLE IV. Experiment with *Ova C. fasciatus*. The relative effect of varied conditions of Temperature and Humidity on
(a) Fertility of eggs laid, (b) Hatching of eggs laid.

[illegible]

TABLE V. *Ova P. irritans. Influence of the temperature and humidity upon hatching of eggs; all eggs laid in incubator "75 Wet."*

Incubator 75 Wet.									
Date	Tube	Box	Jar	Temperature	Humidity	No. examined	No. hatched	Percentage hatched	
1 Oct. 1910	×	—	—	74·1	·79	8	8	100 0/10	
10 „	—	—	×	74·2	·78	49	45	92 0/10	
10 „	×	—	—	74·2	·78	12	7	58 0/10	
*14 „	—	×	—	74·2	·75	23	15	65 0/10	
15 Feb. 1911	×	—	—	74·5	·76	23	17	74 0/10	
15 „	×	—	—	74·5	·76	23	12	52 0/10	
Incubator 85 Wet.									
26 Sept. 1910	—	—	×	85	·74	27	23	85 0/10	
14 Oct. 1910	—	×	—	84	·72	16	11	69 0/10	
Cellar.									
				Max.	Min.				
6 Oct. 1910	—	—	×	58	57	·90 approx.	21	9	43 0/10
*14 „	—	×	—	56	55	·90 „	18	11	61 0/10
Laboratory Cupboard.									
11 Aug. 1910	×	—	—	about 60	·83 approx.	12	9	75 0/10	
1 Oct. 1910	—	—	×	„ 60	·85 „	31	24	77 0/10	
Warm Cupboard.									
				Max.	Min.				
22 Sept. 1910	×	—	—	76	69	·60 approx.	8	3	37 0/10
18 Oct. 1910	—	×	—	73	69	·60 „	12	4	33 0/10

* Signifies a portion of a divided batch of eggs.

TABLE VI. *Ova P. irritans*. Twenty females, fed for one week, were divided into four batches and kept in boxes with a number of males. These batches were then subjected to varied treatment as regards Temperature and Humidity.

Dates	Batch	Number of females	Number of males	Place where fleas were kept	Temperature	Humidity	Number of eggs laid per female per day	Number laid	Hatched	Place where eggs were kept till hatched
18 Aug.—1 Sept.	A	5	Av. 4.7	Incubator 75 Wet	75	.77	1.11	86	42 = 51%	Incubator 75 Wet
2—18 Sept.	A	Av. 4.4	6	" 85 Dry	84.3	.58	1.00	74	Nil	" 85 Dry
19 Sept.—9 Oct.	A	4	6	" 85 Dry	81.8	.58	1.21	102	36 = 35%	" 75 Wet
10 Oct.—1 Nov.	A	3	Av. 8.8	" 75 Wet	74.3	.74	1.55	107	53 = 49%	" 75 Wet
2 Nov.—25 Nov.	A	3	Av. 9.1	" 75 Wet	74.6	.70	1.18	85	35 = 41%	" 75 Wet
18 Aug.—1 Sept.	B	5	Av. 5	Incubator 75 Dry	74.6	.60	1.40	110	48 = 44%	Incubator 75 Dry
2—18 Sept.	B	5	6	Cellar	max. 62 min. 61	.94	1.03	93	25 = 27%	Cellar
19 Sept.—9 Oct.	B	5	6	Cellar	57 56	.93	.43	45	9 = 20%	Incubator 75 Wet
10 Oct.—1 Nov.	B	5	Av. 9.1	Incubator 75 Dry	74.3	.53	1.61	185	108 = 58%	" 75 Wet
2 Nov.—25 Nov.	B	4.1	Av. 8	" 75 Wet	74.6	.70	1.71	171	87 = 50%	" 75 Wet
18 Aug.—1 Sept.	C	5	Av. 4.7	Incubator 85 Dry	83.9	.61	1.54	116	22 = 19%	Incubator 85 Dry
2—18 Sept.	C	Av. 4.2	Av. 5.6	" 75 Wet	75.1	.77	1.58	106	49 = 46%	" 75 Wet
19 Sept.—9 Oct.	C	4	6	" 75 Wet	74.2	.74	1.84	155	66 = 42%	" 75 Wet
10 Oct.—1 Nov.	C	Av. 3.8	Av. 9.1	" 85 Dry	84.5	.61	1.54	135	48 = 35%	" 75 Wet
2 Nov.—25 Nov.	C	3	Av. 9.1	" 75 Wet	74.6	.70	1.9	137	49 = 36%	" 75 Wet
18 Aug.—1 Sept.	D	5	Av. 5	Cellar	max. 63 min. 62	.94	.70	53	25 = 47%	Cellar
2—18 Sept.	D	5	Av. 5.8	Incubator 75 Dry	75.1	.56	1.00	90	20 = 22%	Incubator 75 Dry
19 Sept.—9 Oct.	D	5	6	" 75 Dry	74.8	.51	1.19	125	55 = 44%	" 75 Wet
10 Oct.—1 Nov.	D	5	Av. 9.3	Cellar	max. 56 min. 55	.93	.27	31	4 = 13%	" 75 Wet
2 Nov.—25 Nov.	D	Av. 4.6	Av. 9.6	Incubator 75 Wet	74.6	.70	.98	110	64 = 58%	" 75 Wet

TABLE VI (a). *Ova P. irritans*. Second portion of test. All batches of fleas A, B, C and D kept in Incubator 75 Wet. The ova laid by the several batches were "bulked," then divided into four lots and put under different conditions to hatch.

Place	Period of laying	Temperature	Humidity	Number of eggs	Number which hatched	Percentage which hatched	Aver.
Incubator 43 Dry	26 Nov.—13 Dec.	93·1	·56	81	9	11 0/10	9%
	14 Dec.—3 Jan.	92·9	·57	57	6	10 0/10	
	4 Jan.—16 Jan.	93·0	·55	33	2	6 0/10	
Incubator 75 Dry	26 Nov.—13 Dec.	74·7	·49	81	Nil	—	4%
	14 Dec.—3 Jan.	74·8	·51	57	Nil	—	
	4 Jan.—16 Jan.	74·4	·53	33	7	21 0/10*	
Incubator 75 Wet	26 Nov.—13 Dec.	76·0	·66	81	36	45 0/10	52 0/10
	14 Dec.—3 Jan.	76·1	·66	57	35	61 0/10	
	4 Jan.—16 Jan.	75·2	·74	33	18	54 0/10	
Cellar		max. min.					
	26 Nov.—13 Dec.	47·6 45·8	·91	81	20	24 0/10	26 0/10
	14 Dec.—3 Jan.	49·4 48·3	·93	57	18	32 0/10	
	4 Jan.—16 Jan.	48·6 47·4	·93	33	6	18 0/10	

* Owing to an alteration in Incubator the humidity rose to ·66 for a period of 12 to 20 hours on the 5th Jan. and 4 eggs hatched out of a batch put in on the 3rd Jan. Of subsequent batches 3 eggs hatched with the humidity about ·54 to ·55 for the critical period; the last batch put in on a falling humidity failed to hatch.

TABLE VI (b). *Ova P. irritans. Egg Fertility test (compiled from Table VI). Females put under different conditions to lay eggs; all hatched in incubator 75° Wet.*

Batch	Place of laying	Conditions of laying		No. of eggs per female laid per day	Conditions of hatching		Percentage hatched
		Temperature ° F.	Humidity		Temperature ° F.	Humidity	
A	Incubator 75° Wet	74·3	·74	1·55	74·3	·74	49
B	„ 75° Dry	74·3	·53	1·61	74·3	·74	58
C	„ 85° Dry	84·5	·61	1·54	74·3	·74	35
D	Cellar	55·5	·93	·27	74·3	·74	13

TABLE VII. *Ova X. cheopis. Influence of temperature and humidity upon the hatching of eggs; all eggs laid in incubator 75° Wet.*

Date	Place of hatching	Temperature ° F.	Humidity	Number of eggs	Number hatched	Per-centage	
31 July 1911	Incubator 85 Wet	84·6	·81	25	15	60 0/10	
1 Nov. 1911	„	84·6	·74	21	11	52 0/10	
26 Nov. 1911	„	{ 3 days at 76 4 „ 92·5	{ ·65 ·67	24	11	46 0/10	
3 Jan. 1912	Incubator 93 Wet	92·6	·66	13	1	8 0/10	
12 Oct. 1911	„ 85 Dry	85	·61	18	1	6 0/10	
26 Nov. 1911	„ 93 Dry	93·4	·56	24	3	12 0/10	
3 Jan. 1912	„ 93 Dry	93·2	·56	13	3	23 0/10	
31 July 1911	Incubator 75 Wet	75·6	·84	25	19	76 0/10	
12 Oct. 1911	„	74·7	·76	18	12	66 0/10	
1 Nov. 1911	„	74·8	·70	24	14	58 0/10	
3 Jan. 1912	„	75·5	·71	13	5	38 0/10	
31 July 1911	Incubator 75 Dry	76	·60	25	12	48 0/10	
12 Oct. 1911	„	75	·54	18	3	16 0/10	
26 Nov. 1911	„	74·8	·49	24	Nil	—	
3 Jan. 1912	„	75·2	·50	13	2	15 0/10	
19 Oct. 1911	Warm Cupboard	70	·56	18	3	16 0/10	
26 Nov. 1911	„	61	·58	24	Nil	—	
		Max.	Min.				
31 July 1911	Cellar	64	63	·94	25	17	68 0/10
19 Oct. 1911	„	57	55	·93	18	Nil	—
26 Nov. 1911	„	48	46	·93	24	Nil	—
19 Oct. 1911	Beehive	60	42	—	18	Nil	—
26 Nov. 1911	„	45	34	—	24	Nil	—
26 Nov. 1911	Lab. Cupboard	53	45	·87	24	Nil	—

TABLE VIII. *Ova* X. cheopis. The relative effect of Temperature and Humidity on (a) Fertility of eggs laid, (b) Hatching of eggs laid.

Place	Series I										Series II										Series III												
	Females put in different places to lay and ova allowed to hatch in the same places as laid										Females put into different places to lay. Ova all put into incubator 75° Wet and allowed to hatch										Females all put into incubator 75° Wet to lay. Ova distributed in batches among the different test places												
	Laid and hatched					Hatched					Hatched					Hatched					Hatched												
	Number of females used	Date, 1912	Temperature	Humidity	No. of eggs laid	Percentage per female	Number hatched	Date, 1912	Temperature	Humidity	No. of eggs laid	Percentage per female	Number hatched	Date, 1912	Temperature	Humidity	No. of eggs	Date, 1912	Temperature	Humidity	No. of eggs	Date, 1912	Temperature	Humidity	No. of eggs	Date, 1912	Temperature	Humidity	No. of eggs	Date, 1912	Temperature	Humidity	No. of eggs
Incubator 75° Wet	50	15 June	75·3	·83	15	·30	5 = 33% 7 = 58%	29 July	74·2	·82	38	·76	15 = 39%	25 June	75·0	·86	10	25 June	75·0	·86	10	25 June	75·0	·86	10	25 June	75·0	·86	10	25 June	75·0	·86	10
	50	2 July	75·3	·80	12	·24	7 = 58%	29 July	74·2	·82	38	·76	15 = 39%	9 Aug.	75·0	·81	18	9 Aug.	75·0	·81	18	9 Aug.	75·0	·81	18	9 Aug.	75·0	·81	18	9 Aug.	75·0	·81	18
Incubator 93° Wet	50	15 June	94·0	·81	17	·34	none	29 July	74·2	·82	23	·46	6 = 26%	25 June	95·9	·70	10	25 June	95·9	·70	10	25 June	95·9	·70	10	25 June	95·9	·70	10	25 June	95·9	·70	10
	50	2 July	95·4	·74	2	·04	none	29 July	74·2	·82	23	·46	6 = 26%	9 Aug.	93·4	·78	18	9 Aug.	93·4	·78	18	9 Aug.	93·4	·78	18	9 Aug.	93·4	·78	18	9 Aug.	93·4	·78	18
Incubator 75° Dry	50	15 June	75·9	·61	12	·24	6 = 50%	29 July	74·2	·82	19	·38	4 = 21%	25 June	76·0	·59	10	25 June	76·0	·59	10	25 June	76·0	·59	10	25 June	76·0	·59	10	25 June	76·0	·59	10
	50	2 July	76·0	·59	16	·32	9 = 56%	29 July	74·2	·82	19	·38	4 = 21%	9 Aug.	75·0	·57	18	9 Aug.	75·0	·57	18	9 Aug.	75·0	·57	18	9 Aug.	75·0	·57	18	9 Aug.	75·0	·57	18
Incubator 93° Dry	50	15 June	93·0	·57	17	·34	none	29 July	74·2	·82	30	·60	7 = 23%	25 June	93·0	·55	10	25 June	93·0	·55	10	25 June	93·0	·55	10	25 June	93·0	·55	10	25 June	93·0	·55	10
	50	2 July	93·0	·54	1	·02	none	29 July	74·2	·82	30	·60	7 = 23%	9 Aug.	93·0	·54	18	9 Aug.	93·0	·54	18	9 Aug.	93·0	·54	18	9 Aug.	93·0	·54	18	9 Aug.	93·0	·54	18
Cellar	50	15 June	max. min. 57·6 56·5	·93	12	·24	3 = 25%	29 July	74·2	·82	1	·02	none	25 June	max. min. 58·0 57·2	·93	10	25 June	max. min. 58·0 57·2	·93	10	25 June	max. min. 58·0 57·2	·93	10	25 June	max. min. 58·0 57·2	·93	10	25 June	max. min. 58·0 57·2	·93	10
	50	2 July	58·5 57·7	·93	none	0	none	29 July	74·2	·82	1	·02	none	9 Aug.	57·5 56·5	·93	18	9 Aug.	57·5 56·5	·93	18	9 Aug.	57·5 56·5	·93	18	9 Aug.	57·5 56·5	·93	18	9 Aug.	57·5 56·5	·93	18
Warm Cupboard	50	15 June	66·5	·71	6	·12	1 = 16%	29 July	74·2	·82	21	·42	9 = 42%	25 June	66·7	·69	10	25 June	66·7	·69	10	25 June	66·7	·69	10	25 June	66·7	·69	10	25 June	66·7	·69	10
	50	2 July	64·8	·76	22	·44	5 = 22%	29 July	74·2	·82	21	·42	9 = 42%	9 Aug.	64·6	·69	18	9 Aug.	64·6	·69	18	9 Aug.	64·6	·69	18	9 Aug.	64·6	·69	18	9 Aug.	64·6	·69	18
Laboratory Cupboard	50	2 July	max. min. 67·3 61·0	·81	5	·10	none	29 July	74·2	·82	3	·6	none	25 June	max. min. 61·3 59·6	·80	10	25 June	max. min. 61·3 59·6	·80	10	25 June	max. min. 61·3 59·6	·80	10	25 June	max. min. 61·3 59·6	·80	10	25 June	max. min. 61·3 59·6	·80	10
	50	15 June	70·4 52·3	—	8	·16	2 = 25%	29 July	74·2	·82	14	·28	2 = 14%	9 Aug.	63·4 56·3	·83	18	9 Aug.	63·4 56·3	·83	18	9 Aug.	63·4 56·3	·83	18	9 Aug.	63·4 56·3	·83	18	9 Aug.	63·4 56·3	·83	18
Beehive	50	15 June	70·4 52·3	—	8	·16	2 = 25%	29 July	74·2	·82	14	·28	2 = 14%	25 June	66·8 52·4	—	10	25 June	66·8 52·4	—	10	25 June	66·8 52·4	—	10	25 June	66·8 52·4	—	10	25 June	66·8 52·4	—	10
	50	2 July	64·8	·76	22	·44	5 = 22%	29 July	74·2	·82	21	·42	9 = 42%	9 Aug.	64·2 43·0	—	18	9 Aug.	64·2 43·0	—	18	9 Aug.	64·2 43·0	—	18	9 Aug.	64·2 43·0	—	18	9 Aug.	64·2 43·0	—	18
Ice chest	20	15 June	41·9	·98	none	0	none	25 June	41·7	·98	none	0	none	25 June	41·7	·98	none	25 June	41·7	·98	none	0	none	25 June	41·7	·98	none	0	none	25 June	41·7	·98	none
	20	15 June	41·9	·98	none	0	none	25 June	41·7	·98	none	0	none	25 June	41·7	·98	none	25 June	41·7	·98	none	0	none	25 June	41·7	·98	none	0	none	25 June	41·7	·98	none

TABLE IX. *Ova Ct. canis.* A number of fleas found in the dog's bed. These were placed in a box and put into Incubator 75 Wet. The eggs were divided and batches put into the different Incubators and Cupboards to hatch.

Date	Place	Temperature		Humidity	Number of eggs	Number hatched	Percentage hatched
26 July 1911	Incubator 85 Wet	85·3		·82	10	7	70 %
"	" 75 Wet	76·2		·83	10	8	80 %
"	" 75 Dry	76·3		·60	10	5	50 %
		Max.	Min.				
"	Cellar	64·5	63·4	·94	10	8	80 %
"	Lab. Cupboard	73·0	65·0	·74	10	7	70 %
18 Sept. 1911	Incubator 75 Wet	74·6		·76	17	11	64 %

2. LARVAE.

(i) *Ability of newly hatched larvae to exist without food in case.* In Buckland's *Curiosities of Natural History* (Appendix to Fourth Series) a paper on "Fleas," read by the Rev. J. Hussey before the Ashmolean Society of Oxford in 1836, is given as the authority for the statement that young larvae can exist for from two to three weeks without food.

The following experiments have been carried out to test this point:

METHOD. A number of newly hatched larvae were put away in small tubes with three or four scraps of blotting-paper to afford foothold and cover. Coloured blotting-paper is to be preferred, as it shows up the larvae and renders counting easier. In most of the experiments the tubes were corked, but, in order to test if this made any serious difference to the results, a few tests were made with cotton-wool plugs in place of corks and sand in place of the blotting-paper. Any dead larvae were removed daily to avoid all possibility of the survivors feeding on them. (See Tables X, XI and XII.)

While the results obtained support the correctness of Hussey's statement, the differing conditions of temperature and humidity were also found to have a very marked influence. So different were the results with *C. fasciatus* in mid-winter and *P. irritans* in the spring, that a number of repetitions of the experiment were made in order to test how far the difference was specific and how far a question of season. It appears that *C. fasciatus* is much better adapted to survive unfed than *P. irritans*; in one instance, in the cellar, in the winter the average length of life of a batch of larvae without food was 75 days and some individuals survived over 100 days. (See Table X.) There is an apparent well-marked seasonal change in the powers of endurance of *C. fasciatus*, as shown by a comparison of the average length of life

in the incubators during winter and summer. This apparent lack of endurance of summer hatched larvae is really more marked than at first sight appears, if we consider the fact that the humidity in the incubators is higher in the summer than in the winter¹. Judging, however, by the fact that this seasonable difference is confined to the experiments with incubators and is not traceable in the cellar or warm cupboard records, *it must be concluded that this wide discrepancy is due to the difference in the ventilation of the incubators in summer and winter*. Throughout the whole course of these experiments the ventilators have been open as freely as possible consistent with the required percentage of humidity. This results in keeping the ventilators of the Wet incubators all but shut during the winter.

There is some evidence of seasonal difference in the powers of endurance of *P. irritans* to be gleaned from Table XI. The differences between the March and June series of *P. irritans* are probably due to individual variation and the small numbers used in the March experiments. The results of the later tests with larger numbers only show the influence of favourable or unfavourable conditions of humidity and temperature.

As will be seen from the tables, *X. cheopis* comes between *C. fasciatus* and *P. irritans* as regards its powers of endurance unfed. Tubes with cotton-wool plug and a little sand at the bottom gave a better result than the corked tubes with blotting-paper under moist and moderately cool conditions. With regard to the experiment in incubators 75 Dry on the 15th July, 1912, I am inclined to question the humidity record; the readings for a few days about this period were very irregular and quite abnormal and it is possible that the high records may have been merely snatch readings. If the humidity really averaged 71, and did not sink below 63, it is remarkable that the larvae lived so short a time.

Although *X. cheopis* is normally an inhabitant of hot climates, it is surprising that the newly-hatched larvae of this species are not able to survive hot and dry conditions any better than *C. fasciatus* (see Table XII).

A small trial with *Ct. canis* in July, 1911 (see Table XIII), suggests that this species is scarcely so well adapted to exist unfed as *P. irritans*; it is certainly very inferior in this respect to *C. fasciatus*.

¹ Newly hatched larvae in small tubes might possibly be utilised as a method of testing the conditions of humidity prevailing in corners, holes in walls, etc. where it is not possible to take readings with an hygrometer.

Advantage is apparently taken by *C. fasciatus*, and possibly by *P. irritans* as well, of these powers of prolonged fasting to increase the variability of the brood in the matter of emergence. In some rearing experiments, it was noticed that the larvae when given food did not all commence to grow for varying periods of from several days to two or three weeks and a few larvae became no larger than when they emerged from the egg. Meanwhile their fellows of the same batch had attained their full growth.

To test the crawling powers of the freshly emerged unfed larvae, the tracks of several individuals of *C. fasciatus*, *P. irritans* (Fig. 2, p. 502) and *X. cheopis* (Fig. 3, p. 503) were followed with a pencil on sheets of paper, the tests lasting for 30 minutes in each case.

It will be noted that both *P. irritans* and *X. cheopis* seem better adapted for wandering in search of food than *C. fasciatus*. An attempt was also made with *C. fasciatus* and *X. cheopis* to ascertain if the freshly emerged larvae crawled quite at random or whether they showed any instinctive tendency to progress in the direction of food, moisture or cover.

Eleven newly hatched larvae of *C. fasciatus* were placed on a large sheet of white paper with the following substances arranged in a circle at equal distances from their starting point: (a) a piece of blood-soaked rag, (b) a piece of gauze spotted with flea faeces, (c) a piece of plain cloth, and (d) a pat of moist sand. The larvae crawled in various directions, but none went near the cloth; two approached quite close to the damp sand, but turned aside and wandered past it. Three came quite close to the gauze with the flea droppings on it, but seemed quite uninfluenced by the proximity of this food, and either wandered past it or turned back towards their starting point. None went anywhere near the blood-soaked rag. The only conclusion possible during the period of watching was that their wanderings were quite at random and in no way influenced by the various articles.

The experiment was repeated with *X. cheopis*, the only difference being that the flea-spotted gauze was replaced by dry sand. Fifteen newly hatched larvae were placed in a central circle and watched for 15 minutes, with the following result:

- 5 did not leave the circle.
- 1 reached the wet sand.
- 1 reached the dry sand.
- 1 reached the cloth.

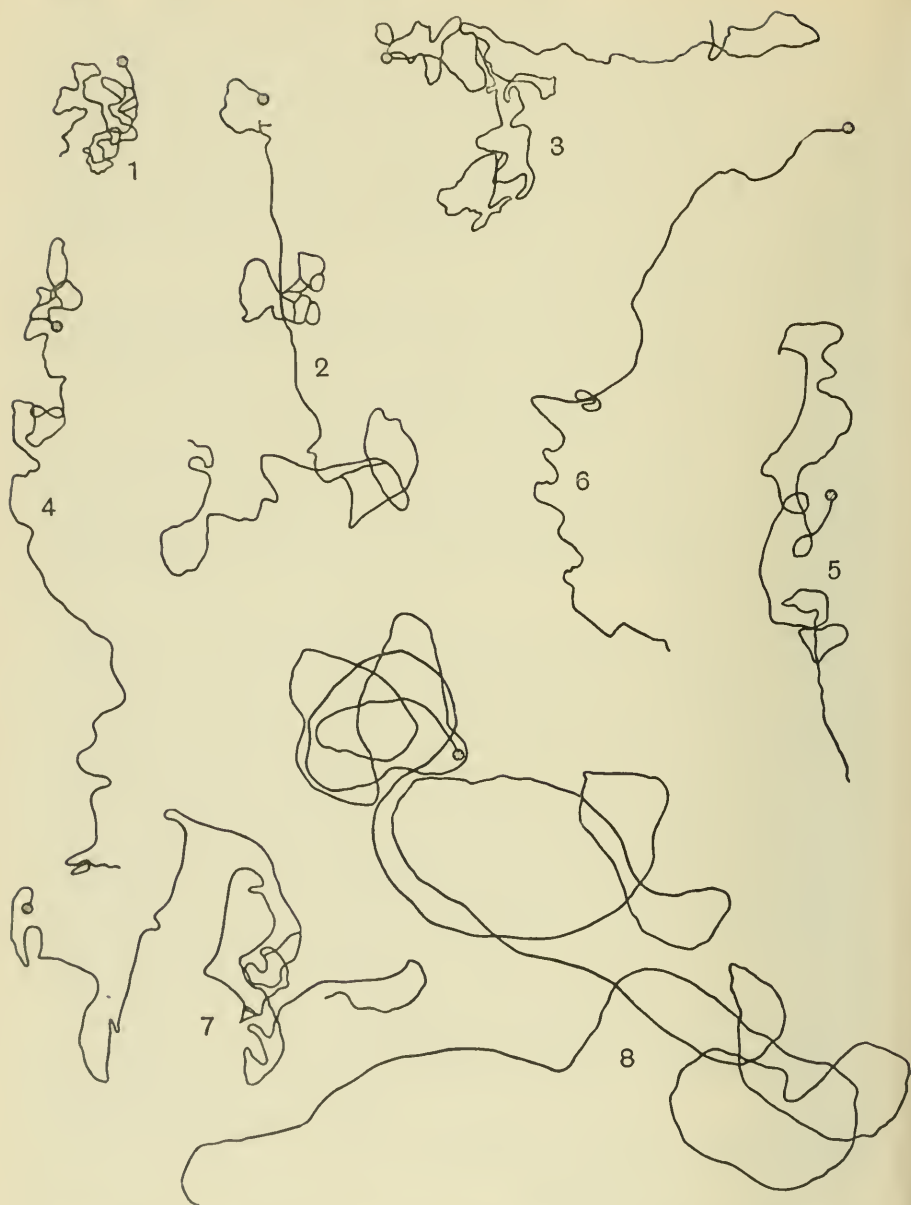


Fig. 2. 1. Larva of *C. fasciatus*. Newly hatched. 30 minutes on white paper. Distance=about 6 inches. 2. Larva of *C. fasciatus*. Newly hatched. 30 minutes on white paper. Distance=about 15 inches. 3. Larva of *C. fasciatus*. Newly hatched. 30 minutes on white paper. Distance=about 16 inches. 4. Larva of *C. fasciatus*. Newly hatched. 30 minutes on white paper. Distance=about 12 inches. 5. Larva of *C. fasciatus*. Newly hatched. On cork carpet 30 minutes. Distance=about 11 inches. 6. Larva of *C. fasciatus*. Newly hatched. On cork carpet 30 minutes. Distance=about 8 inches. 7. Larva *P. irritans*. Newly hatched. On cork carpet 30 minutes. Distance=about 18 inches. 8. Larva of *P. irritans*. Newly hatched. On white paper 30 minutes. Distance=about 42 inches.

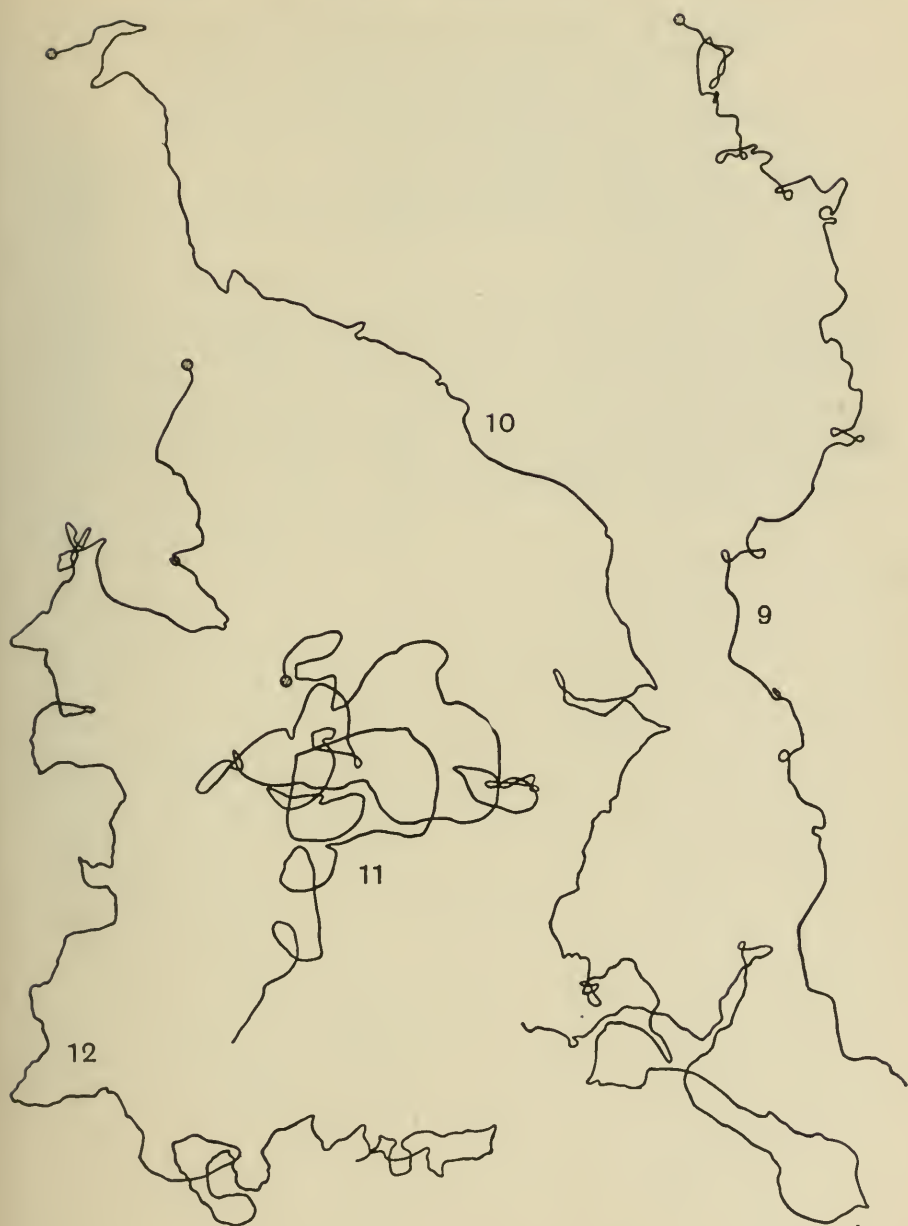


Fig. 3. 9. No. 1 Larva of *X. cheopis*. Newly hatched. On white paper 30 minutes. Distance=about 16 inches. 10. No. 2 Larva of *X. cheopis*. Newly hatched. On white paper 30 minutes. Distance=about 27 inches. 11. No. 3 Larva of *X. cheopis*. Newly hatched. On white paper 30 minutes. Distance=about 23 inches. 12. No. 4 Larva of *X. cheopis*. Newly hatched. On white paper 30 minutes. Distance=about 23 inches.

- 1 stopped to feed just before reaching the blood-soaked rag, presumably on a speck of dried blood which had become detached, as its alimentary canal at once darkened.
- 1 wandered out past and between the wet and dry sand.
- 3 remained sluggish after crawling about midway between the starting circle and the wet sand.
- 1 stopped between the starting circle, blood-soaked rag and the wet sand.

The larvae are, however, very prompt at attacking food in their immediate proximity. A larva has been seen to partake of a speck of flea faeces when placed in the lid of a small cardboard box that was apparently quite clean, but was found to have a few specks of flea droppings on it when carefully examined. It was a matter of a few seconds only for the perfectly white freshly emerged larvae to show a dark line as the food entered its stomach.

TABLE X. *Newly hatched larvae C. fasciatus. Experiment to determine length of life unfed. Series I.*

A. In corked tubes.

Date	Place	Temperature max. min.	Average Humidity	Number put into tubes	Detail		Average life
13 Dec. '10	Cellar	49 48	·93	8	2 dead in	9 days	23 days
					1 "	19 "	
					1 "	25 "	
					2 "	29 "	
					2 "	31 "	
14 Dec. '10	Incubator 75 Wet	74·4	·75	7	2 "	13 "	18 "
					2 "	18 "	
					1 "	20 "	
					2 "	24 "	
17 Dec. '10	Incubator 85 Wet	84·4	·69	8	2 "	10 "	14 "
					6 "	15 "	
18 Dec. '10	Lab. Cupboard	max. min. 53 44	·84	10	1 "	10 "	26 "
					2 "	16 "	
					2 "	26 "	
					2 "	29 "	
					1 "	33 "	
					2 "	39 "	
18 Dec. '10	Incubator 85 Dry	85	·59	10	4 "	3 "	5 "
					4 "	5 "	
					2 "	8 "	
19 Dec. '10	Incubator 75 Dry	76	·43	10	all "	3 "	3 "
19 Dec. '10	Warm Cupboard	64	·57	10	" "	3 "	3 "
19 Dec. '10	Beehive	*max. min. 44 32		10	2 "	13 "	23 "
					7 "	23 "	
					1 "	39 "	
22 July '11	Warm Cupboard	74	·62	12	5 "	2 "	4 "
					3 "	4 "	
					1 "	5 "	
					3 "	7 "	
22 July '11	Beehive	max. min. 85·5 59·5	—	12	11 "	2 "	2·4 "
					1 "	7 "	
23 July '11	Lab. Cupboard	73·3 65·3	·73	12	6 "	2 "	6 "
					1 "	3 "	
					1 "	5 "	
					2 "	10 "	
					1 "	13 "	
					1 "	18 "	
23 July '11	Cellar	64·5 63·5	·94	12	1 "	2 "	26·2 "
					4 "	19 "	
					2 "	24 "	
					2 "	36 "	
					3 "	39 "	

* Average temperature for the last 29 days only.

TABLE X.—*continued.*

Date	Place	Temperature	Average Humidity	Number put into tubes	Detail		Average life
23 July '11	Incubator 75 Wet	75.9	.82	12	2 dead in	3 days	10.8 days
					1 "	4 "	
					2 "	5 "	
					3 "	12 "	
					1 "	16 "	
					2 "	18 "	
					1 "	22 "	
23 July '11	Incubator 75 Dry	77.6	.52	12	9 "	1 day	1.3 "
					3 "	2 days	
23 July '11	Incubator 85 Wet	86	.82	12	5 "	1 day	3.3 "
					1 "	2 days	
					2 "	3 "	
					1 "	4 "	
					2 "	8 "	
					1 "	11 "	
23 July '11	Incubator 85 Dry	84	.68	12	10 "	1 day	1.4 "
					1 "	3 days	
					1 "	4 "	
29 Nov. '11	Incubator 75 Wet	75.8	.65	12	1 "	6 "	20 "
					1 "	13 "	
					3 "	20 "	
					2 "	23 "	
					3 "	24 "	
					1 "	25 "	
					1 "	26 "	
29 Nov. '11	Incubator 75 Dry	75.2	.49	12	all "	2 "	
29 Nov. '11	Incubator 92 Wet	92.5	.67	12	1 "	1 day	5 "
					1 "	3 days	
					1 "	4 "	
					1 "	5 "	
					5 "	6 "	
					3 "	7 "	
29 Nov. '11	Incubator 92 Dry	93.8	.57	12	3 "	1 day	3 "
					3 "	2 days	
					1 "	3 "	
					4 "	4 "	
					1 "	7 "	
11 Dec. '11	Cellar	max. min. Dec. 48.7 47.4 Jan. 47.2 45.9 Feb. 45.8 44.6	.92 .92 .92	9	3 "	57 "	75 "
					2 "	64 "	
					2 "	77 "	
					1 "	101 "	
					1 "	120 "	
24 Dec. '11	Lab. Cupboard	Dec. 53.9 46.2 Jan. 52.5 44.9 Feb. 54.4 45.3	.87 .89 .86	10	2 "	5 "	34 "
					1 "	11 "	
					1 "	32 "	
					3 "	43 "	
					2 "	51 "	
					1 "	53 "	

TABLE X.—*continued.*

Date	Place	Temperature	Average Humidity	Number put into tubes	Detail		Average life
					4 dead in	4 days	5 days
24 Dec. '11	Warm Cupboard	60·4	·62	9	4	6	
					1	7	
25 Dec. '11	Beehive	max. 46·8 min. 35·0 Jan. 43·6 34·0 Feb. 46·7 34·3	—	12	2	5	50
					2	10	
					4	77	
					2	87	
					1	89	
25 Dec. '11	Incubator 92 Wet	92	·71	13	13	1 day	
26 Dec. '11	Incubator 92 Wet	92·5	·71	3	3	1	
B. Placed in small glass tubes containing a little sand and with cotton-wool plugs in place of corks.							
18 Mar. '12	Incubator 75 Wet	75·2	·80	10	2	11 days	17
					2	14	
					2	17	
					3	21	
					1	22	
18 Mar. '12	Incubator 75 Dry	72	·54	10	9	1 day	1 day
					1	2 days	
18 Mar. '12	Incubator 92 Wet	91·5	·68	10	3	4	6 days
					7	7	
18 Mar. '12	Incubator 92 Dry	92	·59	9	6	1 day	1 day
					2	2 days	
					1	3	
18 Mar. '12	Cellar	max. 49·1 min. 48·1 Apr. 50·3 49·1 May 54·2 53·2	·93 ·93 ·93	9	2	17	34 days
					2	22	
					2	29	
					1	39	
					1	59	
					1	78	
18 Mar. '12	Beehive	Mar. 54·1 38·2 Apr. 64·1 37·3	—	10	2	7	29
					1	11	
					1	14	
					2	17	
					1	22	
					1	26	
					1	33	
					1	35	
Mar. '12	Warm Cupboard	57·5	·63	10	all	2	
18 Mar. '12	Lab. Cupboard	max. 54·4 min. 45·5	·86	10	3	3	7
					3	7	
					3	10	
					1	12	

TABLE XI. *Newly hatched larvae P. irritans. Experiment to determine length of life unfed.*

A. In corked tubes.

Date	Place	Temperature		Average Humidity	Number put into tubes	Detail		Average life
15 Mar. '11	Incubator 85 Dry	84		55	1	1 dead in	2 days	2 days
14 Mar. '11	Incubator 75 Dry	75		48	3	1 "	1 day	3 "
						1 "	3 days	
						1 "	5 "	
16 Mar. '11	Incubator 85 Wet	83		70	3	3 "	1 day	1 day
17 Mar. '11	Incubator 85 Wet	83		70	1	1 "	1 "	
13 Mar. '11	Incubator 75 Wet	74		76	6	4 "	5 days	6 days
						2 "	7 "	
18 Mar. '11	Lab. Cupboard	max. min. 56 47		86	2	1 "	12 "	13 "
						1 "	15 "	
19 Mar. '11	Beehive	55	34	—	4	1 "	4 "	19 "
						2 "	24 "	
						1 "	26 "	
20 Mar. '11	Cellar	46	44	92	4	2 "	15 "	18 "
						1 "	19 "	
						1 "	23 "	
21 Mar. '11	Warm Cupboard	61		61	2	1 "	1 day	5 "
						1 "	9 days	
24 Mar. '11	Incubator 75 Wet	75		74	4	4 "	6 "	
3 April '11	Incubator 75 Wet	75		76	8	2 "	4 "	6 "
						5 "	7 "	
						1 "	10 "	
3 April '11	Incubator 85 Wet	84		68	6	1 "	1 day	4 "
						1 "	3 days	
						2 "	4 "	
						1 "	5 "	
						1 "	6 "	
*21 June '11	Cellar	max. min. 58 57		93	12	6 "	9 "	12 "
						5 "	14 "	
						1 "	19 "	
*21 June '11	Lab. Cupboard	63	59	85	12	2 "	5 "	9 "
						9 "	9 "	
						1 "	12 "	
†22 June '11	Incubator 85 Dry	83		68	12	4 "	2 "	3 "
						8 "	3 "	
†22 June '11	Incubator 85 Wet	84		79	12	5 "	4 "	5 "
						5 "	5 "	
						2 "	6 "	
‡26 June '11	Incubator 75 Wet	75		74	12	3 "	3 "	4 "
						8 "	4 "	
						1 "	5 "	
‡26 June '11	Incubator 75 Dry	75		55	12	12 "	1 day	1 day

* Batches of larvae were divided on each date * † ‡.

TABLE XI.—*continued.*

Date	Place	Temperature	Average Humidity	Number put into tubes	Detail	Average life
20 July '11	Incubator 75 Wet	76·5	·82	12	3 dead in 4 days	6 days
					8 " 6 "	
					1 " 8 "	
20 July '11	Incubator 75 Dry	77	·56	12	5 " 2 "	3 "
					4 " 3 "	
					2 " 4 "	
					1 " 5 "	
20 July '11	Incubator 85 Wet	85·8	·83	12	3 " 3 "	4 "
					4 " 4 "	
					5 " 5 "	
20 July '11	Incubator 85 Dry	84	·72	12	4 " 2 "	3 "
					8 " 3 "	
22 July '11	Cellar	max. min. 64 63	·94	12	3 " 3 "	6 "
					2 " 4 "	
					6 " 6 "	
					1 " 14 "	
22 July '11	Lab. Cupboard	75·2 66·7	·70	12	8 " 3 "	4 "
					3 " 6 "	
					1 " 7 "	
22 July '11	Warm Cupboard	75	·60	12	8 " 2 "	2 "
					2 " 3 "	
					2 " 4 "	
22 July '11	Beehive	max. min. 86 59	—	12	1 " 2 "	4 "
					8 " 4 "	
					2 " 5 "	
					1 " 6 "	
29 Nov. '11	Incubator 75 Wet	76·5	·67	11	2 " 2 "	5 "
					4 " 5 "	
					2 " 6 "	
					2 " 7 "	
					1 " 8 "	
29 Nov. '11	Incubator 75 Dry	75·2	·49	11	all " 2 "	
29 Nov. '11	Incubator 92 Wet	92	·66	11	" " 1 day	
29 Nov. '11	Incubator 92 Dry	93·7	·57	11	9 " 1 "	1 day
					2 " 2 days	
2 Dec. '11	Warm Cupboard	64·6	·59	12	1 " 2 "	3 days
					7 " 3 "	
					4 " 4 "	
11 Dec. '11	Cellar	max. min. 48·8 47·7	·93	12	4 " 11 "	14 "
					2 " 14 "	
					2 " 15 "	
					2 " 16 "	
					1 " 18 "	
					1 " 20 "	

TABLE XI.—*continued.*

Date	Place	Temperature max. min.		Average Humidity	Number put into tubes	Detail		Average life
11 Dec. '11	Lab. Cupboard	54·4	47·4	·87	12	3 dead in	8 days	10 days
						9 "	11 "	
12 Dec. '11	Beehive	47·4	35·2	—	12	7 "	4 "	7 "
						2 "	7 "	
						2 "	10 "	
						1 "	23 "	
B. In small glass tubes plugged with cotton-wool and a little sand at bottom.								
1 Jan. '12	Incubator 75 Wet	76·8	·65	10	3 "	2 "	4 "	
					2 "	3 "		
					5 "	5 "		
1 Jan. '12	Incubator 92 Wet	93·5	·68	10	3 "	1 day	2 "	
					3 "	2 days		
					4 "	3 "		
1 Jan. '12	Incubator 92 Dry	94	·54	10	6 "	1 day	1 day	
					4 "	2 days		
1 Jan. '12	Incubator 75 Dry	75·5	·49	10	4 "	2 "	3 days	
					4 "	3 "		
					2 "	4 "		
20 Jan. '12	Cellar	max. min. 46·2 44·5	·92	10	3 "	5 "	9 "	
					5 "	10 "		
					2 "	14 "		
20 Jan. '12	Beehive	43·2	35·1	—	10	7 "	5 "	6 "
					2 "	7 "		
					1 "	9 "		
22 Jan. '12	Warm Cupboard	58·5	·63	10	8 "	2 "	2 "	
					2 "	3 "		
22 Jan. '12	Lab. Cupboard	max. min. 51·1 42·8	·90	10	4 "	4 "	7 "	
					3 "	8 "		
					3 "	12 "		
25 Jan. '12	Incubator 75 Dry	75	·52	10	8 "	1 day	1 day	
					2 "	2 days		
26 Jan. '12	Incubator 75 Wet	75·6	·71	10	6 "	5 "	6 days	
					3 "	8 "		
					1 "	10 "		
29 Jan. '12	Incubator 93 Wet	93	·65	10	4 "	2 "	3 "	
					6 "	3 "		
29 Jan. '12	Incubator 93 Dry	94	·56	10	2 "	1 day	2 "	
					7 "	2 days		
					1 "	3 "		
30 Jan. '12	Beehive	max. min. 40 27	—	10	all "	4 "		
	Daily readings	41 26						
		36 19						
		42 12						
30 Jan. '12	Cellar	40·5	39·4	·91	10	7 "	7 "	9 "
						2 "	14 "	
						1 "	16 "	

TABLE XII. *Newly hatched larvae X. cheopis. Experiment to determine length of life unfed.*

A. In corked tubes.

Date	Place	Temperature max. min.		Average Humidity	Number put in tubes	Detail	Average life
14 June '11	Cellar	57	56	·93	6	2 dead in 12 days	16 days
						2 " 16 "	
						2 " 19 "	
4 Sept. '11	Cellar	60	58	·93	12	2 " 10 "	19 "
						3 " 14 "	
						1 " 16 "	
						2 " 18 "	
						3 " 28 "	
						1 " 30 "	
4 Sept. '11	Lab. Cupboard	67	58	·75	12	3 " 8 "	10 "
						4 " 10 "	
						2 " 11 "	
						1 " 12 "	
						2 " 15 "	
4 Sept. '11	Incubator 75 Wet	74·8		·76	12	3 " 7 "	11 "
						4 " 10 "	
						2 " 12 "	
						1 " 14 "	
						1 " 15 "	
						1 " 26 "	
4 Sept. '11	Incubator 85 Wet	84·9		·79	12	4 " 4 "	6 "
						6 " 6 "	
						2 " 8 "	
28 Sept. '11	Incubator 85 Dry	84·9		·57	12	4 " 4 "	5 "
						7 " 5 "	
						1 " 6 "	
28 Sept. '11	Incubator 75 Dry	75		·50	12	9 " 2 "	2 "
						3 " 3 "	
28 Sept. '11	Warm Cupboard	67		·64	12	12 " 3 "	3 "
11 Dec. '11	Incubator 75 Wet	75·6		·67	7	4 " 11 "	12 "
						1 " 13 "	
						1 " 14 "	
						1 " 15 "	
11 Dec. '11	Incubator 92 Wet	92·1		·67	7	2 " 2 "	4 "
						1 " 4 "	
						2 " 5 "	
						2 " 6 "	
24 Dec. '11	Incubator 75 Dry	74·7		·51	9	1 " 2 "	5 "
						4 " 4 "	
						3 " 6 "	
						1 " 7 "	
13 July '12	Incubator 93 Wet	95·2		·83	8	5 " 2 "	3 "
						2 " 3 "	
						1 " 5 "	
15 July '12	Incubator 93 Dry	93		·69	6	5 " 1 day	1 day
						1 " 2 days	

TABLE XII.—*continued.*

Date	Place	Temperature	Average Humidity	Number put in tubes	Detail			Average life
15 July '12	Incubator 75 Dry	77·5	·71*	6	3 dead in	1 day		1 day
					3	"	2 days	
B. Placed in small glass tubes with a little fine sand; the tubes plugged with cotton-wool.								
4 Aug. '12	Incubator 75 Wet	74·7	·81	6	3	"	13 "	17 days
					3	"	22 "	
4 Aug. '12	Incubator 75 Dry	75	·64	6	5	"	1 day	1 day
					1	"	2 days	
4 Aug. '12	Incubator 93 Wet	93·3	·78	6	2	"	1 day	5 days
					1	"	4 days	
					1	"	6 "	
					2	"	9 "	
4 Aug. '12	Incubator 93 Dry	93	·54	6	6	"	1 day	1 day or less
5 Aug. '12	Warm Cupboard	65·1	·72	4	1	"	2 days	4 days
					1	"	4 "	
					2	"	6 "	
5 Aug. '12	Cellar	Aug. Sept. ^{max. min.} 57·5 56·5	·93	6	2	"	10 "	22 "
					1	"	12 "	
					1	"	29 "	
					2	"	35 "	
15 Aug. '12	Lab. Cupboard	62·4 54·5	·86	6	1	"	5 "	20 "
					1	"	15 "	
					1	"	20 "	
					1	"	25 "	
					1	"	28 "	
					1	"	30 "	
15 Aug. '12	Beehive	64·5 44·7	—	6	1	"	4 "	21 "
					1	"	19 "	
					1	"	22 "	
					1	"	25 "	
					2	"	28 "	

* Humidity very irregular; varied from ·79 to ·63.

TABLE XIII. *Newly hatched larvae Ct. canis. Experiment to determine length of life unfed.*

In corked tubes.

Date	Place	Temperature	Average Humidity	Number put in tubes	Detail			Average life
29 July '11	Incubator 75 Wet	76	·85	10	6 dead in	2 days		4 days
					2	"	4 "	
					1	"	6 "	
					1	"	10 "	
29 July '11	Incubator 75 Dry	77·7	·59	10	7	"	2 "	3 "
					3	"	4 "	
29 July '11	Cellar	^{max. min.} 63 62	·94	10	4	"	3 "	6 "
					5	"	7 "	
					1	"	13 "	

(ii) *Influence of temperature, humidity and nature of the food supply upon rearing.* The chief factors influencing the larval life of the species experimented on appear to be humidity of the air, temperature, and food supply. A situation sufficiently dry to ensure that dust and fine sand do not cling is essential for rearing larvae, while at the same time the percentage of humidity in the air in their immediate proximity should be above a mean of .60 to .65. It is of course probable that owing to local moistening larvae may thrive amid what are otherwise impossible circumstances. For example, the margins of a periodically wetted area in a very dry place would give limited but quite feasible opportunities for larval life; being very active crawlers, the larvae could easily keep to the margins, even if the area fluctuated in size. The numerous instances of larvae quitting their cocoons shortly after spinning, when the latter were subjected to hot, dry conditions, may indicate an instinct on the part of the larva, if an unfavourable change occurs, to search for a more suitable situation for its metamorphosis, even after the cocoon has been formed.

While it is difficult to fix exact limits to the range of temperature to which larvae can adjust themselves, it is certain that unfavourable, but not necessarily fatal conditions, may enormously delay development. Cold delays development in some species, and is fatal to others.

As regards food, the range of possible diet is no doubt a wide one, as already noted in the Committee's Report (Vol. VIII, No. 2, May 1908, *Journal of Hygiene*), but some of my experiments have shown that all the species are not alike in this respect and that some do not succeed on food that gives good results for others.

P. irritans is not so particular about its food as *C. fasciatus*; it can also survive in draughty situations or under conditions of humidity that are fatal to *C. fasciatus*.

X. cheopis appears to resemble *P. irritans*, rather than its congener, in the matter of food, but is more susceptible to cool conditions than the other species dealt with, and possibly better able to resist dry heat. This difference in adaptability on the part of *X. cheopis* and *C. fasciatus* in respect to food has probably a direct relation to the habits of their hosts, assuming, as I do, that the Committee's Report ("On the Bionomics of Fleas," Vol. VIII, No. 2, p. 245, May 1908, *Journal of Hygiene*) is correct in considering *M. rattus* as the host of *X. cheopis* and *M. decumanus* as the host of *C. fasciatus*. *M. rattus* is apparently chiefly to be found in houses, warehouses, granaries, etc., places which would be likely to offer many situations in which larvae might be reared quite apart from their hosts, provided that they could make

shift on a diet devoid of their parents' droppings. On the other hand, *M. decumanus*, as an inhabitant of sewers and cellars, is more associated with the ground level or beneath it and occupies situations which are less likely to afford suitable places for the rearing of larvae apart from the nests of their hosts. The habits of the two fleas fit those of their respective hosts. Compared with *C. fasciatus*, *X. cheopis* is far more closely attached to the rat; accustomed to travel with its host, it can afford to drop some of its eggs with a fair chance of the resulting larvae being reared. Although, under these circumstances, the droppings of the adult fleas are likely to be thinly scattered over a wide area, this need not prove disastrous for rearing the larvae of this species which are not entirely dependent upon food in this form. Supposing that the adults of *C. fasciatus* had the same habit of clinging to the rat that *X. cheopis* has, such a habit would be unduly wasteful, as numbers of ova might fall in places where there would be no chance of a food supply other than the thinly scattered flea droppings.

The cool, and probably more humid, situations in which *M. decumanus* must frequently nest, would make it advantageous for the adult fleas to wait quite a considerable time for the return of their host to the nest, for among the haunts of the animal it is likely to be one of the few favourable situations for flea breeding.

C. fasciatus. The experiments (see Tables XV to XVIII) show that a cool situation with a humid atmosphere is most favourable to the larvae of this species, but that temperature up to 85° F. may be survived by some individuals; low humidities at any temperature will prove fatal to active larvae. Under the conditions of the tests the critical point of humidity was found to be in the neighbourhood of .60 to .65 for temperatures up to 75° F. in a still atmosphere. With a draughty situation a considerably higher humidity is necessary for success in rearing from the egg—note the persistent mortality in the laboratory cupboard, which, in spite of its high percentage of humidity, gave scarcely any successful results (see Exp. h, Table XVI).

Moistening with either water or urine will mitigate a condition of extreme drought in which no larvae can survive for longer than a day or two. If liquid be applied in suitable quantities at regular intervals, breeding is possible. Moistening may, however, be easily overdone if applied injudiciously; there is some possibility that urine is a more effective moistening agent than water¹.

¹ These moistening experiments were instituted as it was thought that the possibility of children urinating within a house or hut with earth floors, or damping of the floor from

From the experiments detailed in Table XV, the nature of the food supplied to larvae even when well grown is seen to be of importance. The fact that, in so humid a place as the cellar, there is so large a difference in the mortality of those nourished on rat faeces 80 % or B.S. rag 40 % respectively, suggests that the difference is really one of nutrition, and is not due to the mechanical difficulty of absorbing dry food from large masses. This point is further emphasised in Table XVI in the series carried out during May, June and July 1911 in the cellar and incubators 85 Wet and 75 Wet respectively, to test the relative values as food of B.S. rag, flea faeces and crushed rat faeces for *C. fasciatus*. The May series (in incubator 75 Wet) (Exp. a, Table XVI) consisted of a batch of young larvae from eggs laid and hatched under precisely similar conditions and then divided on the same date between the experiments. It will be noted that, while the flea faeces and B.S. rag batches showed a mortality of 29 % in both cases, the mortality with rat faeces as food was 100 %. A similar result is also apparent with experiments in the cellar on the 18th and 25th June (Exp. f, Table XVI) and in incubator 85 Wet on the 8th and 13th July (Exp. c, Table XVI), but in these cases the larvae under different dates were from separate layings.

When nourished on different diets, the speed with which larvae attain the cocoon stage varies considerably, conditions other than food being similar. The results obtained are somewhat contradictory, in some cases flea faeces and at others B.S. rag showing an advantage. These discrepancies are probably due to more than one cause. Firstly, the innate variability of the species must be taken into account and, secondly, the food supplied was not properly standardised. Flea faeces, which consist of the dejecta of *P. irritans* on the gauze coverings of the boxes in which they are kept, have possibly been given in varying quantities, it being no easy matter to gauge the exact quantity, and the B.S. rag has differed in quality, that is, in regard to the amount of blood on it. Three different cloths have been used since the commencement of the experiments, the second being decidedly inferior, while the last was much the best. It was not, unfortunately, anticipated that difference in quality of food would be of such great importance to

any cause might make all the difference between the extermination or survival of flea larvae during the dry season. The urination of rats, especially nestling rats, will certainly be an important factor in the relative scarcity or abundance of fleas in hot, dry climates. The factor of local moistening is of course applicable when fleas are reared on caged rats.

the larvae as now appears probable in the light of the experiments. The rationale of the matter seems to be that the best food gives the quicker development as well as the lowest mortality.

P. irritans. The results with this species largely resemble those obtained with *C. fasciatus* as regards conditions of temperature and humidity, but at the same time there is evidence of its being a hardier species, with greater ability to survive in hot and dry conditions. This is most clearly shown by its survival under cool, draughty conditions in the laboratory cupboard (see Exp. *g*, Table XXI), in which place hardly one larva of *C. fasciatus* was reared (see Exp. *h*, Table XVI). Further testimony is available from the hot Wet incubator series of the two species, a few individuals of *P. irritans* having been reared at a temperature of 93° F. (see Exp. *c*, Table XXI, Jan. 25 and Mar. 22, 1912).

The experiments of feeding on rat faeces and bran show that this species is also more adaptable in the matter of food. Probably the advance in human comfort and cleanliness will have gradually forced the larvae of *P. irritans* to abandon dependence they may once have had upon a diet having origin in the blood of their parents' host.

X. cheopis. A low percentage of humidity in the air is as fatal to this species as it is to *C. fasciatus* and *P. irritans*. Table XXV shows that the conditions in incubators 85 Dry and 75 Dry are quite as impossible for it as for the other species dealt with, although there is some hint (see warm cupboard under date of 4th September 1911, and incubator 85 Dry 28th August 1911 (Table XXV)) that the larvae have greater powers of endurance than those of *C. fasciatus* and *P. irritans*, and the evidence afforded by the tests applied to newly hatched unfed larvae is, on the whole, of a confirmatory character (see Table XII). On the other hand, the tests of transferring well grown larvae of the rat fleas from the cages to incubators to complete their metamorphosis are hardly in agreement (compare Table XIX with Table XV), and suggest that *C. fasciatus* is even better adapted to the exigence of a sudden rise in temperature than its congener. This is not, however, necessarily contradictory to the view that *X. cheopis* is better adapted for breeding under hot conditions than *C. fasciatus*, but rather that the latter is better able to resist a sudden change.

In Table XXV where comparison is made of the results obtained with *C. fasciatus*, *P. irritans* and *X. cheopis* under similar conditions it will be noticed that there is a lower mortality among *X. cheopis* reared in incubator 85 Wet and 75 Wet, which is reversed in the

cooler situations. It is evident that cold, or even cool, conditions are very fatal to *X. cheopis*. The cellar, laboratory cupboard and beehive experiments in Table XXV, as well as Table XIX, dealing with the transfer of last instar larvae from the cages, show clearly that *X. cheopis* is ill adapted to survive the cold of English autumn conditions.

Ct. canis (Table XXIV). Sufficient ova of this species were obtained from a dog's bed to permit of a small experimental test upon rearing the larvae under varied conditions of temperature and humidity.

The larvae were all hatched from eggs laid in incubator 75 Wet and then distributed in batches as usual. The species would appear to be very intolerant of extreme conditions during its active larval life and, so far as it is safe to judge from this one experiment, cool and draughty situations are most suitable for its development.

MITES. Attempts to feed larvae on oatmeal, or to use oatmeal in place of sand with other foods, failed with *C. fasciatus* (see Table XVI under date of 14th February), and similar attempts with *P. irritans* in incubator 75 Wet, 85 Wet, the cellar and laboratory cupboard, on a more elaborate scale, gave like results. In all cases the jars developed swarms of mites, and the larvae disappeared, leaving no trace of remains.

Two direct tests were made in order to see if the mites were responsible for the failure.

With *P. irritans* two tubes were prepared with the necessary sand and B.S. rag. Into one numbers of mites and 11 newly hatched larvae were placed; in the other a few mites only and 12 half-grown larvae; both tubes were kept in incubator 75 Wet. Within nine days all trace of the newly hatched larvae had disappeared. The tube containing the half-grown larvae produced nine fleas.

With *C. fasciatus* the following experiment was carried out: half-grown larvae were taken from the cages and a batch of 14 placed in each of two tubes prepared as follows: tube No. 1 contained sand, oatmeal and B.S. rag, and was quite free from mites; tube No. 2 contained exactly the same, but with the addition of swarms of mites. The quantity of food was large in order that there might be no question of scarcity of food. Twelve fleas were reared from tube No. 1 and twelve from tube No. 2, the average time in both cases being 19 days.

The factors of extermination would appear therefore to be the mites, but they appear to be inimical only to flea larvae while they are young; most probably the flea larvae are destroyed when moulting, as the latter are then too sluggish to resist or to get away.

TABLE XV. *Development of larvae, C. fasciatus. Influence of temperature, humidity and food supply upon larvae taken from the breeding cages when half to full grown.*

Date	No of larvae	Receptacle	Place	Food	Temp.	Humidity	No. of cocoons found	No. of fleas reared	Mortality	No. of days start to emergence	No. of days in cocoon	Remarks
5 July '10	19	Card jar	Incubator 85° Wet	Rat faeces	85°	about .72	—	1	95%	14	—	
6 "	15	"	"	"	75°	" .67	—	1	93	20	—	
7 "	14	"	Lab. cupboard	"	about 60	" .84	—	2	86	1 took 26	—	
					max. 55.9 min. 54.6	"				1 " 29		
9 "	20	"	Cellar	"		" .93	—	4	80	1 " 27	1 took 50	1 active larva seen 73 days after start
						"				1 " 31		
						"				1 " 63		
						"				1 " 125		
3 Aug. '10	18	"	Warm cupboard	"	69.9 67.8	" .60	2	4	78	2 " 12	1 " 13	
						"				1 " 16	1 " 15	
						"				1 " 19	1 " 30	
8 "	25	"	Incubator 85° Wet	B. S. Rag	84.8	" .72	8	18	24	3 " 9		
						"				2 " 13		
						"				2 " 17		
						"				4 " 20		
						"				2 " 21		
						"				1 " 22		
						"				1 " 30		
						"				1 " 35		
						"				1 " 40		
						"				1 " 51		
9 "	21	"	Incubator 75° Wet	"	74.3	" .69	5	19	10	1 " 10	1 " 10	
						"				1 " 13	3 " 11	
						"				12 " 16	1 " 12	
						"				3 " 19		
						"				2 " 20		
						"				1 " 34	1 " 24	
						"				4 " 36	1 " 25	
1 "	19	"	Lab. cupboard	"	about 60	about .84	4	15	21	2 " 41	1 " 28	
						"				2 " 43		
						"				2 " 45		
						"				1 " 50		
						"				1 " 53		
						"				1 " 35		
2 "	16	"	Cellar	"	max. 59.0 min. 57.9	about .93	1	9	44	2 " 38		
						"				1 " 49		
						"				4 " 51		
						"				1 " 55		
3 "	15	"	Warm cupboard	"	70.2 67.8	about .60	—	0	100	2 " 16		2 active larvae seen 27 days after start
5 "	23	"	Incubator 75° Wet	"	74.5	" .70	—	13	44	2 " 24		
						"				2 " 26		

5	"	25	"	Lab. cupboard	"	"	about 60	about '84	14	19	24	2	"	23
												2	"	25
												1	"	27
												2	"	29
												1	"	31
												1	"	34
												3	"	35
												2	"	42
												1	"	46
												1	"	49
												1	"	53
												2	"	59
												1	"	26
												4	"	38
												2	"	40
												2	"	49
												2	"	54
												1	"	59
												2	"	75
												1	"	81
Sept. '10	23		"	Lab. cupboard	"	"	about 60	about '84	—	15	35			
Aug. '10	13						up to Nov. approximate							
"	9						60	'84						
"	8													
Sept. '10	20		"	Lab. cupboard	"	B.S. Rag	from Nov. to close of expt.		9	58	30			
"	23						max. min.							
"	10						54.9 44.5	'84						
Av. date, 1														
31 Aug. }	83													
Dec. '10	14		Tube	Beehive		B.S. Rag			11	9	36			
						Jan.	43.9 32.0					1	took 148	
						Feb.	43.8 30.7					1	" 152	4 cocoons opened
						March	52.5 33.3					2	" 135	30 Sept. '11 con-
						April	61.7 36.8					1	" 131	tained 2 living
						May	72.8 45.2					1	" 153	pupae, 2 empty.
						June	73.0 56.5					1	" 178	Remaining co-
						July	80.3 58.6					1	" 211	coons opened 16
						Aug.	80.2 57.6					1	" 218	Nov. '11, all were
						Sept.	75.3 48.0					1	" 267	empty
						Oct.	61.9 41.3							
Sept. '12	22		Card jar	Incubator 75 Dry		B.S. Rag	75.0	'52	—	—	100			
"	22		"	" 93 Dry		"	92.0	'53	—	—	100			1 larva attempted to spin

Approximate
Shortest max. 26
Average 45
Longest min. 114

(a) Incubator 75° Wet.

Date	Tube	Box	Jar	Food	No. of larvae	Temperature	Humidity	Length of larval life to cocoon stage (in days)			Maximum number of days after hatching during which larvae were observed	No. of fleas reared	Mortality %
								Min.	Aver.	Max.			
30 July '10	x	—	—	B.S. Rag	2	75	unrecorded	—	—	—	6	0	100
5 Aug. '10	x	—	—	"	4	75	.66	—	—	—	25	0	100
11 " " '10	—	x	—	"	6	75	.70	—	—	—	3	0	100
24 Sept. '10	—	—	x	"	16	75	.72	20	21	22	50	8	50
30 " " '10	—	—	x	Dead flies (<i>M. domestica</i>)	6	—	—	9	12	17	—	6	Nil
16 Oct. '10	x	—	—	Rat faeces	15	74.3	.75†	—	14	—	—	1	93
19 " " '10	—	—	—	B.S. Rag	9	74.3	.69†	—	—	—	—	0	100
30 " " '10	x	x	—	"	13	74.2	.74	13	15	18	—	13	13
30 " " '10	x	—	—	Rat faeces	15	74.2	.74	15	16	18	—	8	38
21 Dec. '10	x	—	x	B.S. Rag	28	74.3	.75	19	25	39	—	8	72
14 Feb. '11	x	—	—	*Oatmeal	10	74.4	.76	—	—	—	23	0	100
14 " " '11	x	—	—	*Oatmeal and B.S. Rag	12	74.4	.76	—	—	—	20	0	100
14 Mar. '11	x	—	—	*Oatmeal and flea faeces	12	74.4	.76	—	—	—	59	5	81
14 Mar. '11	x	—	—	Rat faeces crushed	26	75	.80	20	28	45	—	20	29
24 April '11	x	—	—	Powdered blood, no sand	8	74.7	.83	—	24	—	—	4	50
23 May '11	—	—	x	Flea faeces	28	75.8	.84	15	18	20	—	34	29
23 " " '11	—	—	x	Rat faeces crushed	28	75.8	.84	15	17	20	—	20	8100
23 " " '11	—	—	x	B.S. Rag	28	75.8	.84	15	17	22	—	20	29
14 Nov. '11	—	—	x	Bran	22	75.0	.69	—	—	—	32	0	100

Moistening test (see also Table XVII).

(b) Incubator 75° Dry.

Date	Tube	Box	Jar	Food	No. of larvae	Temperature	Humidity	Length of larval life to cocoon stage (in days)			Maximum number of days after hatching during which larvae were observed	No. of fleas reared	Mortality %
								Min.	Aver.	Max.			
13 Jan. '11	x	Moistened	—	B.S. Rag	13	74.4	—	21	24	33	37	8	38
13 " " '11	x	Not moistened	—	"	14	74.4	.75	—	—	—	35	0	100
21 Dec. '10	—	—	x	B.S. Rag	14	76.5	.41	—	—	—	—	0	100
15 Mar. '11	x	—	—	Rat faeces crushed	20	74.5	.49	—	—	—	—	0	100
28 Aug. '11	—	—	x	B.S. Rag	11	74.5	.61	—	—	—	—	0	100

No larvae seen after emergence
No larvae seen after placing in tube
1 larva seen after 2 days

(c) Incubator 85° Wet.

Date	Tube	Box	Jar	Food	No. of larvae	Temperature	Humidity	Length of larval life to cocoon stage (in days)			Maximum number of days after hatching during which larvae were observed	No. of fleas reared	Mortality %
								Min.	Aver.	Max.			
30 July '10	x	—	—	B.S. Rag	4	85	dropped from .80 to .68	—	—	—	5	0	100
4 Aug. '10	x	—	—	"	4	85	.74	—	—	—	—	4	Nil
10 " " '10	—	x	—	"	1	85	.74	—	—	—	—	0	100
9 Sept. '10	—	x	—	Bran	3	85	.70	—	—	—	—	0	100
22 " " '10	—	—	x	B.S. Rag	12	85	.70	—	—	—	—	5	59
13 Oct. '10	x	—	—	Rat faeces	9	84	.72	—	—	—	12	0	100
5 Nov. '10	x	—	—	"	14	84	.72	—	—	—	8	0	100
24 Dec. '10	x	—	x	B.S. Rag	16	84	.68	—	—	—	17	0	100
15 Mar. '11	x	—	—	Rat faeces crushed	26	84	.71	19	22	33	35	6	77
8 July '11	—	—	x	B.S. Rag	20	85.1	.83	9	14	23	—	22	24
8 " " '11	—	—	x	Rat faeces crushed	28	85.1	.83	—	—	—	12	0	100
13 " " '11	—	—	x	Flea faeces	28	85	.82	—	—	—	—	5	82
14 Nov. '11	—	—	x	Bran	22	85.2	.73 for 9 days .750 .69 for 7 days	14	19	26	17	—	100

		(e) Warm Cupboard.		(f) Cellar.		(g) Beehive.		(h) Laboratory Cupboard.			
		Max. Min.						Approximate only until Nov. '10			
24 Dec. '10	Crushed Rat faeces	×	—	×	—	×	—	×	—	×	—
15 Mar. '11	B.S. Rag	×	—	×	—	×	—	×	—	×	—
28 Aug. '11		×	—	×	—	×	—	×	—	×	—
6 Aug. '10	B.S. Rag	×	—	×	—	×	—	×	—	×	—
10 Oct. '10	"	×	—	×	—	×	—	×	—	×	—
22 " '10	Rat faeces	×	—	×	—	×	—	×	—	×	—
12 Nov. '10	B.S. Rag	×	—	×	—	×	—	×	—	×	—
29 Mar. '11	Rat faeces crushed	×	—	×	—	×	—	×	—	×	—
31 July '10	B.S. Rag	×	—	×	—	×	—	×	—	×	—
4 Aug. '10	"	×	—	×	—	×	—	×	—	×	—
14 " '10	"	×	—	×	—	×	—	×	—	×	—
7 Oct. '10	"	×	—	×	—	×	—	×	—	×	—
22 " '10	Rat faeces	×	—	×	—	×	—	×	—	×	—
4 April '11	"	×	—	×	—	×	—	×	—	×	—
25 June '11	" crushed	×	—	×	—	×	—	×	—	×	—
18 " '11	Flea faeces	×	—	×	—	×	—	×	—	×	—
25 " '11	B.S. Rag	×	—	×	—	×	—	×	—	×	—
25 " '11	"	×	—	×	—	×	—	×	—	×	—
21 Aug. '11	Flea faeces	×	—	×	—	×	—	×	—	×	—
21 " '11		×	—	×	—	×	—	×	—	×	—
10 April '11	Rat faeces crushed	×	—	×	—	×	—	×	—	×	—
25 July '10	B.S. Rag	×	—	×	—	×	—	×	—	×	—
6 Aug. '10	"	×	—	×	—	×	—	×	—	×	—
12 " '10	"	×	—	×	—	×	—	×	—	×	—
18 " '10	Sweat soaked flannel	×	—	×	—	×	—	×	—	×	—
17 Sept. '10	Rat skin	×	—	×	—	×	—	×	—	×	—
27 " '10	B.S. Rag	×	—	×	—	×	—	×	—	×	—
15 Oct. '10	Rat faeces	×	—	×	—	×	—	×	—	×	—
19 " '10	B.S. Rag	×	—	×	—	×	—	×	—	×	—
26 " '10	Rat faeces	×	—	×	—	×	—	×	—	×	—
29 Mar. '11	" crushed	×	—	×	—	×	—	×	—	×	—
13 July '11	Flea faeces	×	—	×	—	×	—	×	—	×	—
13 " '11	B.S. Rag	×	—	×	—	×	—	×	—	×	—
33 " '11	Rat faeces crushed	×	—	×	—	×	—	×	—	×	—

When two or more experiments bear the same date it may be taken that a batch of eggs or newly hatched larvae were divided.

Experiments given in Table XVI, up to and including those of 14 February, 1911, form a continuation of those made with ova set forth in Table II;

The humidity was very low for a portion of the time, from the 21 to 24 Oct. 1910 it averaged .61 only.

The oatmeal developed swarms of mites. No sand was used.

7 cocoons were opened and found to be empty, the larvae must have emerged again after spinning and died.

8 fleas bred. 1 cocoon, opened 8 May 1911, contained a resting larva which died. Remaining cocoons empty (false cocoons); mortality is really nil.

TABLE XVII*. *Development of larvae, C. fasciatus. Influence of temperature and humidity upon larvae reared from egg; all eggs laid in incubator "75 Wet."*

(a) Incubator 75 Wet.

Date	Tube	Box	Jar	Food	No. of larvae	Temperature	Length of larval life to cocoon stage (in days)			Maximum number of days after hatching during which larvae were observed	No. of fleas reared	Mortality
							Min.	Aver.	Max.			
1 Dec. '10	x	—	—	B.S. Rag	11	75	—	—	—	22	0	100%
6 "	x	—	—	"	18	75	—	—	—	17	0	100
8 "	x	—	—	"	14	75	—	—	—	No record	0	100
+28 "	Batch of eggs divided	x	{	Urine at intervals	23	74	15	20	22	40	3	87
+28 "				Water	21	74	15	22½	38	43	5	76
+4 Mar. '11	Batch of 72 eggs divided	{	x	Water 1 c.c. daily	13	74	—	10	—	14	1	92
+4 "				Urine "	25	74	—	17	—	27 (only 2 cocoons found)	12	52

(b) Incubator 75 Dry.

8 Dec. '10	x	—	—	"	12	74	—	—	—	No larvae seen after emergence	0	100
17 "	x	—	—	"	21	74	—	—	—	"	0	100
+3 Jan. '11	Batch of 42 eggs divided	{	x	Urine at intervals	12	76	—	—	—	"	0	100
+3 "				Water "	13	76	—	—	—	"	0	100
+31 "	Batch of 61 eggs divided	{	x	Urine daily	21	77	—	—	—	"	0	100
+31 "				Water "	20	76.5	20	21	23	"	1	95
+23 Feb. '11	Batch of 60 eggs divided	{	x	1 c.c. Urine daily	7	75.5	—	—	—	20	0	100
+28 "				1 c.c. Water "	17	75.5	—	—	—	11	0	100
+17 May '11	—	—	x	0.5 c.c. Urine daily	20	75	—	—	—	28	0	100
+17 "	—	—	x	0.5 c.c. Water "	20	75	—	—	—	1	0	100
6 "	—	—	x	0.5 c.c. Urine "	20	76	—	—	—	No larvae seen after the 1st day	0	100

(c) Incubator 85 Wet.

1 Dec. '10	x	—	—	"	12	84	—	—	—	14	0	100
8 "	x	—	—	"	7	84	—	—	—	20	0	100
+30 "	Batch of 61 eggs divided	{	x	Urine at intervals	24	84	15	15	17	29	5	79
+30 "				Water "	16	84	—	—	—	26	0	100
+24 Feb. '11	Batch of 72 eggs divided	{	x	Urine 1 c.c. daily	19	84	12	14	16	17	2	90
+24 "				Water "	21	84.3	12	16	19	40	2	91

(d) Incubator 85 Dry.

+25 Nov. '10	×	Batch of 41 eggs {	13	84	Urine at intervals	—	—	No larvae seen after hatching	0	100
+25 "	×	divided	8	84	Water	—	—	"	0	100
1 Dec. '10	×	—	11	84	Water at start	—	—	"	0	100
6 "	×	—	15	83	"	—	—	"	0	100
8 "	×	—	9	84	"	—	—	"	0	100
17 "	×	—	19	84	"	—	—	"	0	100
+30 "	×	Batch of 42 eggs {	20	84.9	Urine at intervals	—	—	"	0	100
+30 "	×	divided	15	85	Water	—	—	No larvae seen after hatching	0	100
+27 Jan. '11	×	Batch of 39 eggs {	4	85	Urine daily	—	—	"	0	100
+27 "	×	divided	9	85	Water	—	—	"	0	100
+21 Feb. '11	×	Batch of 50 eggs {	hatching	84	1 c.c. Urine daily	—	—	"	0	100
+21 "	×	divided	unrecorded	84	1 c.c. Water	24	25	"	2	40
6 May '11	—	—	20	84	0.5 c.c. Urine daily	16	18	"	12	40
15 "	—	—	20	84	0.5 c.c. Water	—	—	"	3	85

(e) Warm Cupboard.

9 Nov. '10	×	Batch of 64 eggs {	5	60	Urine at intervals	—	—	18	0	100
+ 3 Feb. '11	×	divided	12	65	Urine daily	—	—	20	0	100
+ 3 "	×	Batch of 70 eggs {	19	63	Water	—	95 +	—	1	94
+ 8 Mar. '11	×	divided	12	60.7	1 c.c. Urine daily	—	—	14	0	100
+ 8 "	×	—	16	61.2	1 c.c. Water	28	30	37	10	38
5 May '11	—	—	20	62.6	0.5 c.c. Urine daily	24	25	26	4 larvae died after spinning	3
6 "	—	—	20	64.6	0.5 c.c. Water	31	36	49	2	80

* The experiments given in Table XVII, up to and including those of 8 March 1911, form a continuation of those made with ova set forth in Table I; data as to the corresponding cocoon stage will be found in Table XXIX.

+ against two experiments of the same date signifies that both were made with specimens from a single batch of ova or newly emerged larvae.

+ One fully fed larva wandered about for 47 days before spinning cocoon.

(iii) LAGGING LARVAE. (See Table XXVI.) In addition to the ability of newly hatched flea larvae to exist for several days or weeks without food, some individuals of *C. fasciatus* were noticed to "lag" when they had attained their full growth, either finishing their larval feeding with extreme slowness or else waiting for considerable periods after they had finished feeding before spinning their cocoons.

It is possible that the larval period is not only lengthened by these practices in a few isolated instances, but that it is generally rendered more variable than would otherwise be the case.

When rearing from the egg, this phenomenon of "lagging" is not very noticeable owing to the varying speed attained in development by different individuals, but it becomes quite evident when a number of full sized larvae are taken from the cages. This was done between the end of January and the middle of March 1911 in order to obtain a number of newly emerged fleas to stock a new cage. About 120 larvae of *C. fasciatus* were put into a jar in incubator 75 Wet (temperature 74°F., humidity 74) on the 27th January, 1911. Several had spun their cocoons by the following day; many fleas emerged and were added to the new cage. On the 4th March it was decided to add a fresh batch of larvae from the cages to the same jar. When it was opened for this purpose some of the larvae put in on the 27th January were discovered to be still active after a period of 36 days, numbers of their fellows having in the meantime emerged as fleas. After putting in the second batch on the 4th of March, active larvae were observed in this jar until the 5th of May; allowing that these were all members of the second batch, it would show "lagging" on the part of some of them for varying periods up to 62 days.

A similar instance occurred with another batch of 100 larvae put into the same incubator on the 17th March; most of these spun at once, but stragglers were seen at intervals, the last being observed on the 19th April, over a month later.

A third series of 100 larvae, put into incubator 75 Wet on the 22nd March (temperature 75°F., humidity 74), showed the same phenomenon, the last active larva being seen on the 1st May, 49 days later. Advantage was taken of one of the "complex transference" cocoon experiments to follow the matter up with a little more detail (see Table XXVI), from which it appears that these lagging larvae are not simply pining individuals that subsequently die, for, as will be seen, most of them eventually spun cocoons and emerged.

TABLE XVIII. *Development of larvae, C. fasciatus reared from the egg; eggs all laid in incubator "75 Wet."*

(a) Influence of varied situation (temperature and humidity); nature of the food supply being maintained constant. Eggs taken from the same batch.

Date	Place (Incubator 75 Wet Lab. cupboard)	Receptacle Box Box	Food B. S. Rag "	No. of larvae	Lengths of larval life to cocoon stage (in days)			Max. number of days after hatching during which larvae were observed	No. of fleas reared	Mortality 100%
					Temp.	Humidity	Min. Aver. Max.			
Oct. '10	{ Warm cupboard { Cellar	Box Box	B. S. Rag "	9 5	75 60 max. min.	Av. 69* 84	— —	— 17	0 0	100 100
Oct. '10	{ Warm cupboard { Cellar	Tube	Rat faeces	5	69 64 48 47	Unrecorded	—	No larvae seen after hatching	0	100
Dec. '10	{ Incubator 85 Wet { " 85 Dry	" "	B. S. Rag	12	84	Moistened	91 to 93	114	0	100
Dec. '10	{ " 75 Wet { " 85 Dry	" "	" "	11 18	84 75	" "	—	No larvae seen after hatching	0	100
Dec. '10	{ " 75 Wet { " 85 Dry	" "	" "	15 12	83 74	" "	—	No record No larvae seen after hatching	0	100
Dec. '10	{ " 85 Dry { " 85 Wet	" "	" "	9	84	" "	—	" "	0	100
Mar. '11	{ " 75 Wet { " 85 Wet	" "	Rat faeces crushed	26	75 84	80 71	20 28 45 19 22 33	59 35	5 6	87 77
Mar. '11	{ " 85 Dry { " 75 Dry	" "	" "	17 20	83 8 74 5	59 49	—	2	0	100
Mar. '11	{ Warm cupboard { Lab. cupboard	" "	" "	26 26	61 5 max. min. 53 3 45 8	62 83	—	No larvae seen after placing in tube " "	0 0	100 100
May '11	{ Incubator 75 Wet { " "	Jar "	Plea faeces Rat faeces	28 28	75 8 75 8	84 84	15 18 20 15 17 20	— 34	20 0	29 100
June '11	{ Cellar { " "	" "	B. S. Rag Rat faeces crushed	28 26	75 8 62 5 61 4 max. min.	84 93	15 17 22 —	— 73	20 2	29 92
Aug. '11	{ " "	" "	" "	27	61 1 59 9 61 5 63 4 Aug. 60 6 59 5	93 91 93	26 27 32 22 27 36	— —	26 13	3 7
July '11	{ Lab. cupboard { " "	" "	Plea faeces B. S. Rag	14 28	72 64 70 62	73 74	22 31 45 —	— 14	10 0	28 100
	{ " "	" "	Rat faeces crushed	28	70 62	74	—	Died off in less than 8 days	0	100

(b) Influence of varied food supply under constant conditions of temperature and humidity in case of eggs or newly hatched larvae taken from the same batch.

* Humidity ran very low for a portion of the time, from 21—24 Oct. it averaged only 61.

are breeding cages when they are free grown.

Date on which larvae were placed under test conditions	No. of larvae	Receiptacle	Place	Food	Temp.	Humidity	No. of cocoons found	No. of fleas reared	Mortality	No. of days start to emergence	No. of days in cocoon	Remarks
30 Nov. '11	22	"	Incubator 75 Wet	B.S. Rag	75.8	.65	21	22	nil	1-16 6-19 3-22 2-24 5-26 1-28 2-32 2-34	—	No larvae seen alive after the start
30 Nov. '11	22	"	Incubator 75 Dry	"	74.7	.51	—	—	100%	—	—	No larvae seen alive after the start
30 Nov. '11	22	"	Incubator 92 Wet	"	92.4	.66	15	13	41	2-13 6-16 5-14 2-19 3-22	6-11 5-14 2-17	2 cocoons contained dried up fleas, remainder false cocoons
30 Nov. '11	22	"	Incubator 92 Dry	"	92.8	.57	13	10	54	3-13 5-16 2-19	6-11 4-14	2 cocoons contained dried up fleas, 1 a dried up larva, remainder false cocoons
5 Dec. '11	22	"	Cellar	"	48.7	.92	1	—	100	—	—	Cocoon opened 12 Apr. '12, found to contain living section of larva which subsequently died, 129 days
5 Dec. '11	22	"	Lab. cupboard	"	54.2 46.0	.84	1	— at least	95	—	—	Cocoon opened, contained dead pupa
2 Apr. '12	22	"	Cellar	"	50.3 49.1	.93	1	—	100	—	—	Larvae dead by 12 Apr. Larvae dead in 2 days
2 Apr. '12	22	"	Lab. cupboard	"	60.6 50.4	.83	1	—	100	—	—	Larvae dead in 2 days
2 Apr. '12	22	"	Beehive	"	64.1 37.3	—	—	—	100	—	—	Larvae dead in 2 days
2 Apr. '12	22	"	Warm cupboard	"	61	.61	—	—	100	1-18 3-21 9-25	1-13 1-14 10-17	Larvae dead in 2 days
2 Apr. '12	22	"	Incubator 75 Wet	"	75.8	.79	21	19	13	2-27 3-35 1-38	4-19 2-21 1-20	Larvae dead in 2 days
2 Apr. '12	22	"	Incubator 75 Dry	"	74.4	.56	—	—	100	—	—	Larvae dead in 2 days
2 Apr. '12	22	"	Incubator 92 Wet	"	93.1	.69	17	10	54	1-11 3-16 3-21 2-25 1-27	1-9 3-16 1-15 2-16 1-18 2-19	Larvae dead in 2 days
2 Apr. '12	22	"	Incubator 92 Dry	"	93.7	.61	16	4	81	2-18 1-21 1-25	2-16 1-19 1-23	Larvae dead in 2 days
9 Sept. '12	22	"	Incubator 75 Dry	"	75.0	.52	—	—	100	—	—	No larvae seen alive after the 9th day

reared from the egg; all eggs laid in incubator "75 Wet."

Date on which eggs were laid	Tube	Box	Jar	Food	Number	Temperature		Humidity		Length of larval life to cocoon stage (in days)			Max. number of days after hatching during which active larvae were observed	Number of Fleas	Mortality		
						max.	min.	Max.	Min.	Average	Max.						
Cellar.																	
6 June '11	×	—	—	B.S. Rag	6	June 58.1	56.7	.93	58	71	84	(2 cocoons)	1	83%			
3 July '11	—	—	×	Flea faeces	14	July 61.6	60.4	.93	{	44	46	65	(13 cocoons)	8	42		
3 July '11	—	—	×	Rat faeces	14	Aug. 64.5	63.1	.94		37	42	44	(14 cocoons)	11	21		
21 Sept. '11	—	—	×	B.S. Rag	22	Sept. 60.6	59.5	.93		74 days	No larvae seen after 4 Dec.	—	100				
						Oct. 56.3	54.8	.92									
						Nov. 50.7	48.9	.91									
						Dec. 48.7	47.4	.92									
Incubator 85 Wet.																	
11 Aug. '11	—	—	×	B.S. Rag	13	84.4		.80	14	16	22	—	12	8			
11 Aug. '11	—	—	×	Flea faeces	11	84.3		.80	—	14	—	—	7	36			
4 Sept. '11	—	—	×	B.S. Rag	22	84.5		.74	12	13	16	(22 cocoons)	20	9			
Incubator 75 Wet.																	
4 Sept. '11	—	—	×	B.S. Rag	22	74.8		.76	14	19	21	(21 cocoons)	21	5			
Incubator 75 Dry.																	
28 Aug. '11	—	—	×	B.S. Rag	11	74		.61	—	—	—	No larvae seen after 1st day	—	—			
Incubator 85 Dry.																	
28 Aug. '11	—	—	×	B.S. Rag	11	84		.60	—	—	—	No larvae seen after 3rd day	—	—			
Warm Cupboard.																	
4 Sept. '11	—	—	×	B.S. Rag	22	68.1		.65	—	—	—	No larvae seen after 7th day	—	100			
Laboratory Cupboard.																	
4 Sept. '11	—	—	×	B.S. Rag	22	max. 65.7	min. 55.7	.77	(Lowest reading 40 on 22 Nov. and three times in Dec. before the 11th)							100	
						Sept. 59.0	50.9	.84									
						Oct. 51.9	46.1	.85									
						Dec. 52.7	44.3	.88									
Beehive.																	
20 Sept. '11	—	—	×	B.S. Rag	22	Sept. 75.3	48.0		(Lowest reading 28° F. on 26 Oct. '11, twice below 40 during Sep.)							100	
						Oct. 61.9	41.3										
Incubator 85 Wet.																	
14 Nov. '11	—	—	×	Bran	22	10 days at 85.2		.73	—							2	90
						7		.69									
Incubator 75 Wet.																	
22 Nov. '11	—	—	×	Bran	22	75.0		.69	20 days							18	18

* The continuation of these experiments through the cocoon stage will be found in Table XXXIV.

TABLE XXI*. *Development of larvae, P. irritans. Influence of temperature, humidity and food supply upon larvae reared from the egg.*

(a) Incubator 75 Wet.

Date on which eggs were laid	Tube	Box	Jar	Food	No. of larvae 7 eggs, no record of hatching	Temp. 75	Humidity Unrecorded	Length of larval life to cocoon stage (in days)			Max. number of days after hatching during which active larvae were observed	No. of fleas reared	Mortality
								Min.	Av.	Max.			
July '10	x	—	—	Rat faeces	4	75	Unrecorded	15	16	18	17	2	—
" '10	x	—	—	Dead flies†	7	75	"	12	15	17	17	2	50% ₁₀
Aug. '10	x	—	—	B.S. Rag	7	75	-66	22	23	26	—	4	43
Oct. '10	—	—	x	"	5	74.3	-72+	—	12	—	13	1	80
"	—	—	x	"	45	74.3	-78+	—	—	—	6	0	100
"	—	—	x	"	15	74.3	-74+	—	—	—	—	0	100
Feb. '11	x	x	—	Bran	17	74.4	-76	—	—	—	No larvae seen after hatching	0	100
"	x	x	—	{ Oatmeal & flea faeces	6	74.4	-76	—	—	—	23	0	100
"	x	x	—	{ Oatmeal and B.S. Rag§	12	74.4	-76	—	—	—	20	0	100
Mar. '11	x	x	—	Rat faeces crushed	13	75	-80	—	—	—	23	1	92
Apr. '11	x	x	—	Powdered dry blood, no sand	7	74.7	-83	14	19	23	—	10	23
June '11	—	—	x	B.S. Rag	24	75.7	-85	10	15	26	18	0	100
"	—	—	x	Flea faeces	24	75.8	-85	15	21	46	—	21	79
Oct. '11	—	—	x	B.S. Rag	22	74.4	-73	13	16	23	—	16	27

(b) Incubator 75 Dry.

June '11	—	—	x	B.S. Rag	20	78	-63	—	—	—	No larvae seen after start	0	100
June '11	—	—	x	Flea faeces	24	75	-53	—	—	—	"	0	100
Aug. '11	—	—	x	B.S. Rag	26	75.7	-59	—	—	—	3 larvae seen on 2nd day, none afterwards	0	100

(c) Incubator 85 Wet.

July '10	x	—	—	B.S. Rag	13 eggs, no record of hatching	about 85	Doubtful record, varies from .80 to .68	12	—	—	—	1	—
Aug. '10	x	—	—	"	12 eggs	85	.74	9	—	—	—	1	—
Sept. '10	—	x	—	Flea faeces	23	85	-70	15	22	27	27	4	83
Oct. '10	—	x	—	Bran	7	84	-72	No cocoons found		8	8	2	72
Mar. '11	x	—	—	Rat faeces crushed	15	84	-71	9	12	15	—	8	47
June '11	—	—	x	B.S. Rag	26	84	-80	8	14	24	—	16	38
"	—	—	x	Flea faeces	20	84	-80	12	14	17	—	8	60
Nov. '11	—	—	x	Bran	22	85.2	-73	—	—	—	15	1	95
Jan. '12	—	—	x	B.S. Rag	24	92.6	-66	11	12	15	—	1	95
Mar. '12	—	—	x	"	22	92.3	-67	10	11	12	—	9	59

(d) Incubator 85 Dry.

June '11	—	—	x	B.S. Rag	26	85	-61	—	—	—	No larvae seen after start	0	100
"	—	—	x	Flea faeces	20	85	-50	—	—	—	"	0	100
Aug. '11	—	—	x	B.S. Rag	26	83.8	-63	—	—	—	"	0	100

[illegible]

* Experiments given in Table XXI up to and including those of 15 February 1911, form a continuation of those made with ova set forth in Table V; data as corresponding cocoon stage will be found in Table XXXVI.

+ *Musca domestica*.

|| Larvae came out, wandered about and failed to pupate.

Temperature and humidity approximate only to Nov. 1910, see actual records after this date in relation to extended larval life.

* 1 cocoon opened 1 Jan. 1911 contained living flea.

1 cocoon opened 1 pup. 1st contained living larv.

TABLE XXII. *Development of larvae, P. irritans reared from the egg. Influence of varied situation (temperature and humidity), nature of food supply being kept constant; in each experiment all eggs taken from the same batch.*

Date on which eggs were laid	Place	Receptacle	Food	Number of larvae	Temperature	Humidity	Length of life, to cocoon stage (in days)			Maximum number of days after hatching during which larvae were observed	Number of fleas reared	Mortality 100 %
Oct. '10	Incubator 75 Wet		Box	15	Dry 74.3	.74	—	—	—	—	0	100 %
Eggs divided						from 21 May '61 only						
Aug. '10												
Eggs divided												
which larvae placed under conditions												
June '11	Incubator 85 Dry	Jar	"	26	85	.61	—	—	No larvae seen after start	—	0	100
	" 85 Wet	"	"	26	84.1	.78	8	14	24	—	16 (2 cocoons empty)	38
	Cellar	"	"	24	max. min. 60.8 59.5	.93	48	54	73	—	16	42
	Lab. Cupboard	"	"	24	68.4 60.1	.77	39	47	54	—	7	71
	Warm Cupboard	"	"	26	73	.62	—	—	No larvae seen after start	—	0	100
	Beehive	"	"	26	max. min. 79 56	—	—	—	" "	" "	0	100
	Incubator 85 Wet	"	Flea faeces	20	84	.79	12	14	17	—	8 (1 cocoon opened empty)	60
	" 85 Dry	"	"	20	85	.49	—	—	No larvae seen after start	—	0	100
	" 75 Wet	"	B.S. Rag	24	75.7	.85	10	15	26	—	21	13
	" 75 Wet	"	Flea faeces	24	75.8	.85	15	21	46	—	5	79
	" 75 Dry	"	"	24	75	.55	—	—	No larvae seen after start	—	0	100
	Cellar	"	"	28	—	—	—	—	—	—	16	42*
	Lab. Cupboard	"	"	28	max. min. 69.0 61.1	.77	30	39	58	—	13	54
	Warm Cupboard	"	"	20	65	.59	—	—	No larvae seen after start	—	0	100
	Beehive	"	"	20	max. min. 68.6 46.3	—	—	—	2	—	0	100

* For conditions of temperature and humidity see record in Table XXI.

TABLE XXIII. Development of larvae *C. gallinae* reared from the egg; eggs obtained from adults kept in incubator "75 Wet" and fed daily on human blood.

Date	Receptacle	Food	Place	Number	Temp.	Humidity	Length of larval life to cocoon stage (in days)			Mortality	Duration of cocoon period	No. of fleas reared
							Min.	Aver.	Max.			
24 March '11	Tube	Dust from nest and flea faeces	Incubator 75 Wet	16	Mar. 74·3 April 75·0 May 74·9	·76 ·78 ·85	13	14	21	Nil	1 in 6 days 4 in 9 days 2 in 11 days 1 in 12 days 1 in 13 days 1 in 14 days 1 in 16 days	11 5 larvae were taken as specimens

TABLE XXIV. Development of larvae *Ct. canis*. Influence of temperature and humidity; eggs obtained from a dog's bed and hatched in incubator "75 Wet."

Date	Tube	Box	Jar	Food	Number	Temperature	Humidity	Length of larval life to cocoon stage (in days)			Max. number of days after hatching during which active larvae were observed	No. of fleas	Mortality
								Min.	Aver.	Max.			
9 May '12	—	—	×	B.S. Rag	22	75·6	·86	12	13	19	—	8	63 9/10
"	—	—	×	"	22	95·1	·75	—	11	—	—	0	100
"	—	—	×	"	22	93·3	·58	—	—	—	5 days	0	100
"	—	—	×	"	22	75·0	·66	—	—	—	1 day	0	100
"	—	—	×	"	22	*—	—	49	61	142	52 days	2	91
"	—	—	×	"	22	*—	—	35	39	42†	38 days	13	40
"	—	—	×	"	22	max. min. 74·3 54·3	—	—	—	—	3 days	0	100
"	—	—	×	"	22	May 39·3 June 40·9 July 43·6	·95 ·99 ·95	—	—	—	75 "	0	100

* See page 461 for records of temperature and humidity, May to August 1912.

† 2 fleas emerged 15 July '12; 2 living fleas were taken out of cocoon on 6 Sept.; 9 living fleas were taken out of cocoon on 1 Oct. 1912.

TABLE XXV*. Development of larvae, comparing influence of temperature and humidity upon larvae of the three species *X. cheopis*, *P. irritans*, and *C. fasciatus*.

Date 1911	Receiptacle	Species	Place	Food	Number	Temperature	Length of larval life to cocoon stage (in days)			Max. number of days after hatching during which active larvae were observed	No. of fleas	Mortality %
							Humidity	Min.	Aver. Max.			
28 Aug.	Jan	<i>X. cheopis</i>	Incubator 85 Dry	B.S. Rag	11	84	.60	—	—	No larvae after 3rd day	—	100
29 "	"	<i>P. irritans</i>	"	"	11	84	.60	—	—	" " 2nd "	—	100
28 "	"	<i>C. fasciatus</i>	"	"	11	84	.60	—	—	" " start	—	100
28 "	"	<i>X. cheopis</i>	Incubator 75 Dry	"	11	74	.61	—	—	" " 1st day	—	100
29 "	"	<i>P. irritans</i>	"	"	11	75	.58	—	—	" " "	—	100
28 "	"	<i>C. fasciatus</i>	"	"	11	74.6	.59	—	—	" " "	—	100
4 Sept.	"	<i>X. cheopis</i>	Incubator 85 Wet	"	22	84.5	.74	12	13	3rd day	20	9
4 "	"	<i>P. irritans</i>	"	"	22	84.7	.77	10	12	"	19	13
11 "	"	<i>C. fasciatus</i>	"	"	21	84.9	.77	9	13	"	14	33
4 "	"	<i>X. cheopis</i>	Incubator 75 Wet	"	22	74.8	.76	14	19	"	21	5
4 "	"	<i>P. irritans</i>	"	"	22	74.9	.75	12	18	"	17	22
14 Oct.	"	<i>C. fasciatus</i>	"	"	22	74.4	.71	13	20	"	20	9
4 Sept.	"	<i>X. cheopis</i>	Warm Cupboard	"	22	68.1	.65	—	—	No larvae after 7th day	—	—
4 "	"	<i>P. irritans</i>	"	"	22	69	.71	—	—	" " 2nd "	—	—
6 "	"	<i>C. fasciatus</i>	"	"	22	67	.66	—	—	" " "	—	—
						max. min.						
4 "	"	<i>X. cheopis</i>	Lab. Cupboard	"	22	Sept. 65.7	.77	—	—	97	—	100
5 "	"	<i>P. irritans</i>	"	"	22	Oct. 59.0	.84	45	62	192	5	77
5 "	"	<i>C. fasciatus</i>	"	"	22	Nov. 54.9	.85	56	62	(4 cocoons)†	—	81
						Dec. 52.7	.88	—	—			
20 "	"	* <i>X. cheopis</i>	Beehive	"	22	Sept. 75.3	—	—	—	37	0	100
19 "	"	<i>P. irritans</i>	"	"	22	Oct. 61.9	—	—	—	188	—	100
19 "	"	<i>C. fasciatus</i>	"	"	22	Sept. 60.6	.93	83	88	(7 cocoons)§	5	68
21 "	"	<i>X. cheopis</i>	Cellar	"	22	Oct. 56.3	.92	37	52	74	—	100
25 "	"	<i>P. irritans</i>	"	"	22	Nov. 50.7	.91	37	48	184 (6 cocoons)*	4	81
25 "	"	<i>C. fasciatus</i>	"	"	22	Dec. 48.7	.92	37	48	(18 cocoons)**	9	36

* In compiling this table, the records for *X. cheopis* are all taken from Table XX and two records for *C. fasciatus* (those in incubators 85 Dry and 75 Dry) from Table XVI. The remaining figures given for *C. fasciatus* and those for *P. irritans* are the results of special experiments not quoted elsewhere.

† 3 July, 1 cocoon opened contained resting larva; 6 Sept., 2 cocoons opened contained resting larvae, 367 days; 11 Sept., 1 cocoon opened contained resting larva, 372 days.

‡ No larvae seen subsequently to 27 Oct. 1911. Lowest reading 28° F. on 26 Oct. 1911. Thermometer was on two occasions below 40° F. during Sept.

§ Last flea emerged 15 June 1912; 4 July, a cocoon opened contained resting larva which pupated on 3 Sept. 1912, 350 days; 11 Sept. 1912, 1 cocoon opened contained a living pupa, 358 days.

|| No larvae seen subsequently to 4 Dec. 1911.

** 6 fleas emerged up to 6 Sept. 1912; 3 fleas emerged 11 Sept. 1912 upon the cocoons being disturbed; cocoons opened 11 Sept. 1912, 5 living pupae, 2 dead pupae, 2 empty.

TABLE XXVI. *Lagging. Full grown larvae, C. fasciatus taken from the cages.*

Date	Number	Place	Receptacle	Temperature	Humidity	Active larvae observed	Cocoons found
11 April '11	26	Incubator 75	Wet Tube	74.7	.87	Several 5 May = 24 days 8 " = 27 " 10 " = 29 " Two 12 " = 31 "	12 in 2 days 3 in 4 " 1 in 5 " 1 in 31 " 2 in 34 "
"	26	Beehive	Tube	max. 65 min. 39	—	One 22 " = 41 "	Only one cocoon found within 8 days
26 April '11	Several	Incubator 75	Wet Tube	74.5	.83	Several 8 " = 12 " 10 " = 14 " 12 " = 16 " 15 " = 19 "	No record. Pupae taken out of cocoons for specimens
11 April '11	26	Cellar	Tube	max. 48.5 min. 46.6 April 53.9 May 52.6 June 58.1 56.7	.91 .92 .93	18 " = 37 " One 8 June = 58 " 15 " = 65 "	1 in 2 days 3 in 6 " 9 in 11 " 3 in 14 " 1 in 16 " 2 in 42 " 5 in 46 " 1 in 58 " 1 in 76 "

3. COCOONS (Tables XXVIII to XLIV).

i. *Influence of temperature, humidity and previous history upon duration of this period.*

In the life history of the flea the cocoon stage is the period in which it is most independent of external conditions of temperature, humidity, etc. and in least danger from the attacks of enemies. It is, therefore, not surprising to find that there tends to be an accumulation of individuals in this stage, from which the active adult population is recruited when suitable conditions offer. In the cocoon period, as in other stages, external conditions and individual idiosyncrasy combine to produce an inextricably interwoven condition of affairs. An attempt was made to disentangle the several strands by means of three different series of experiments with each species:

a. "Continuous" experiments in direct continuity with previous egg and larval experiments:—The cocoons were kept until the emergence of fleas in the same situations as the eggs were laid and hatched and the larvae reared. In the case of *C. fasciatus* these experiments are to be found in Tables XXVIII and XXIX, the corresponding observations on larval and egg stages being given in Tables XVI, XVII and I and II respectively. The cocoon observations for *X. cheopis* set forth in Table XXXIV correspond to Table XX (larvae) and those for *P. irritans* in Table XXXVI to Tables XXI (larvae) and V (ova). In this series the observations upon cocoons are necessarily more limited than the corresponding ones dealing with both eggs and larvae, for in many cases the drastic conditions to which the last-named were subjected left no survivors to undergo a cocoon stage. In nature it is usual for larvae to develop and to spin cocoons before the onset of climatic conditions, which would be fatal to them in the free state.

b. "Simple transference" experiments in which cocoons were obtained as follows:—In case of *C. fasciatus* and *X. cheopis* full-grown larvae were taken from the breeding cages and placed in card jars with sand and B.S. rag. The jars were all put into incubator 75 Wet and the larvae allowed to spin. The cocoons as found were transferred to glass bottomed boxes and these were buried in sand and placed in the different situations investigated. Table XXXI deals with *C. fasciatus* and Table XXXV with *X. cheopis*. In Table XXXVIII are given the results of a series of experiments with *P. irritans*, arranged to correspond

as nearly as possible with those of the other two species. In this instance, however, the larvae were reared until full grown in incubator 75 Wet. In this series of experiments the influence of varied conditions prior to spinning is eliminated as far as possible, and the results afford a comparison, uncomplicated by other circumstances, of the influence of varied external conditions upon the cocoon stage itself.

c. "Complex transference experiments" in which cocoons were maintained under conditions which were the opposite of those under which they were spun. For example, a batch of larvae taken from the breeding cages (in case of rat fleas) or reared in incubator 75 Wet (in the case of *P. irritans*) was placed to spin in incubator 85 Dry, after which the cocoons were transferred to the cellar. In other cases, cocoons spun in the cellar were maintained till emergence of the adult in incubator 85 Dry, and so on. In the tables dealing with this third series (XXXII in case of *C. fasciatus* and XXXIX in case of *P. irritans*) the number of days recorded as being passed in the cocoon stage is approximate¹ only, as the cocoons were not all changed into their new quarters on the same day.

In some of the experiments of the "continuous" series during 1910 and the early months of 1911 discrepancies will be noted between the numbers of cocoons investigated and the fleas recovered. These differences came about owing to the habit the flea larva possesses of sometimes forsaking its cocoon and wandering away to seek a more suitable situation. The deserted cocoons do not differ in any outward respect from full ones and constitute a source of possible error in estimating the mortality at the cocoon stage. So soon as this habit was discovered, deserted cocoons were subjected to careful examination at the close of an experiment, in order to determine whether they were really empty or contained the cast larval skin. The mortality in all but the earliest experiments is based upon the number of cocoons in which actual remains of dead larvae, pupae or fleas were found in the cocoon. In a few of the 1910 experiments, however, made before this routine examination had been instituted, the calculated percentage of failures in the cocoon stage may be too high owing to the inclusion of deserted cocoons. In the "continuous" series the error will consist in attributing too great mortality to the cocoon stage and too little to the larval stage.

¹ For instance, if two cocoons were transferred to the test situation on 1st January and two more on the 10th January, and one flea emerged on February 1st, it would be reckoned to have spent 30 days in the cocoon.

In the second and later series of experiments there is possibly an erroneous estimate of the number of cocoons experimented with. On the whole it was considered better to underestimate the mortality, rather than overestimate it and therefore, in making the calculation, empty cocoons that retained no trace of a tenant were disregarded. They are referred to in the tables as "false" cocoons, and in some cases, where the numbers seemed unduly large, the percentage of mortality has been put down as doubtful. This caution is thought necessary because in hot and dry situations there is a possibility that the larva may leave its cocoon and failing to find a more suitable situation may break up into unrecognizable fragments among the sand and other debris from the cocoons.

Further sources of error in obtaining cocoon statistics are the possibility of two larvae spinning their cocoons against one another and the rare chance of a double cocoon being formed. In only one or two instances, however, has the recorded number of cocoons been exceeded by the joint number of fleas emerging and of remnants found. There is still the possibility that in these cases a wrong count was made.

*P. irritans*¹. The results of the "continuous" series of experiments with this species are given in Tables XXXVI and XXXVII. In the "simple transference" (Table XXXVIII) and "complex transference" experiments (Table XXXIX) the larvae used were reared until full grown in incubator 75 Wet.

For full comprehension of the degree of variability displayed by the cocoons of this species in their reaction to external conditions, it is necessary to study in detail the four Tables referred to above, but the following paragraphs may be found useful in giving a general review of the results obtained.

The influence of high temperature in shortening the duration of the cocoon stage may be seen in the following summary compiled from Table XXXIII.

Experiments, in which the average humidity was between .7 and .9.

Temperatures	Average humidity	Number of cocoons	Average duration of cocoon stage
83.9—85.0	} from .7 { to .9 {	77	11.9
74.4—76.1		78	17.2

¹ The experiments with *P. irritans* are discussed first because with this flea the nature of the reaction to temperature and humidity appears to be less complex than in the case of *C. fasciatus*.

When the range of temperature was at a lower level, the divergence in cocoon period corresponding to difference of temperature was much greater: compare experiments in incubator 75 Dry, 15th April, 1911 and warm cupboard, 19th April, 1911 (Table XXXVIII).

Temperature	Average humidity	Number of cocoons	Average duration of cocoon stage
75·2	·50	4	14 days
63·6	·60	5	44 „

A still greater contrast is shown in the experiment of 11th August, 1910 (Table XXXVII) in which the average duration of the cocoon stage is compared in the case of individuals reared throughout in incubator 75 Wet and the laboratory cupboard respectively.

Temperatures	Average humidity	Number of cocoons	Average duration of cocoon stage
75° F	·60	4	12 days
53 (mean)	·83	6	116 „

As regards humidity it is possible that the increase of ·10 in the second instance given above may be a factor in extending the cocoon period; it is probable, however, that it is unimportant and masked by the normal variation of the individual cocoons. A comparison of the different experiments in Table XXXVIII shows somewhat contradictory evidence in regard to the effects of humidity. For example, in the case of experiments in incubators 85 Dry and 85 Wet, the shorter duration of the cocoon period corresponds to the higher humidity, while a like comparison of the results in 75 Wet and 75 Dry shows that at this temperature the shorter duration usually occurs under the drier conditions. Further, if we compare the records of 75 Wet among themselves we find longer periods corresponding to lower humidity. The conclusion to be drawn is that, in comparison with humidity, temperature and individual variation are the important factors.

There is some evidence suggesting that change to cooler conditions about the spinning period is important in lengthening the cocoon stage, apart from the temperature during this stage¹. At least this is a plausible explanation of the divergent results obtained with broods reared in the laboratory cupboard from the egg stage onwards and those transferred from a warm incubator. Two batches of newly hatched larvae were put into the laboratory cupboard on the 3rd and 12th June

¹ Work by Merrifield on the Seasonal Dimorphism of some species of Lepidoptera shows that an otherwise latent tendency to lengthen the pupal period may be made in response to a change of temperature chiefly if not only at some one time in the larval life. *Experimental Entomology. Factors in Seasonal Dimorphism*, F. Merrifield, F.E.S. Extrait 1^{er} Congrès International d'Entomologie, Bruxelles, 1910.

1911. They had reached the cocoon stage by the 12th July and 12th August respectively, and both batches had an average cocoon period of 17 days, see Table XXXVI. On the other hand in a batch of 41 cocoons transferred from incubator 75 Wet to the Laboratory cupboard on the 30th May 1911 (Table XXXVIII (c)) the average cocoon period of the 32 specimens that emerged, was 53 days. In the latter case the cocoon period began earlier than with the batches put in on the 3rd and 12th June, it continued over the same period, and, in the case of some individuals, was prolonged later. It seems probable that the explanation of this result is that *P. irritans* has a seasonal habit¹, passing the cold months in the cocoon period if possible. A fall of temperature of any moment, as at the approach of autumn, occurring during the late larval or early cocoon stage, brings into action some inherited tendency to lengthen the cocoon period. The length of this stage is due to individual variation as well as to the actual conditions experienced. In this connection it should also be noticed that *change* from a lower to a higher temperature near the date of spinning was also followed by a marked lengthening of the cocoon period.

Below are summarised two interesting experiments from Table XXXIX. In Exp. (a), under date 27th April, 1911, the cocoons were spun in the cellar and shortly afterwards transferred to incubator 85 Dry; in Exp. (b) (May 2nd, 1911) the cocoons suffered a less drastic change: spun in the laboratory cupboard, they were afterwards moved to incubator 75 Dry. In this case the average duration of the cocoon period was only 17 days, whereas in Exp. (a) it was 58 days.

	Temperature at spinning	Humidity at spinning	Transferred to temperature	Humidity	Average duration of cocoon period
(a)	54.1	.92	84.1	.6	58 days
(b)	58.0	.78	75.3	.53	17 „

The following experiments also give good illustration of the influence of a sudden fall in temperature in lengthening the cocoon period. In the "complex transference" experiment under date 2nd May, 1911, Table XXXIX, cocoons were spun in incubator 85 Dry, at a temperature of 84.3 F. and average humidity .59; they were then transferred to the cellar with a mean temperature of about 57 F. during the course of the experiment. The average cocoon period was 48 days. In one

¹ There is perhaps some indication of a seasonal habit in the fact that the November cocoons reared late in the autumn tend to show a higher mortality than in the spring and summer (see Table XXXVIII, experiments in incubator 75 Wet).

of the continuous experiments, the material was kept in the cellar throughout from the egg stage (Table XXXVI under date 3rd June, 1911) at a mean temperature of about 62° F., humidity .90. In this case the individuals passed on an average only 19 days in the cocoon. The inference appears to be that the susceptibility to sudden fall in temperature is, or may be, continued into the early period of cocoon life.

C. fasciatus. With *C. fasciatus* we find a much more complex state of affairs. Individual variation occurs as in the case of *P. irritans*. There is also conclusive evidence of lengthy resting within the cocoon under conditions of high as well as low temperature and over a wide range of humidity. This may be a simple temperature effect or due to change in temperature or both.

A close but general survey of the records obtained convinced me that there was evidence both of an aestivating and an hibernating habit in this species. Extremes both of heat as well as cold produce an effect which is partly of a direct nature and partly, perhaps, a stimulus which calls into action an inborn predisposition to prolonged rest within the cocoon under unfavourable conditions. As was found in the case of *P. irritans*, it is the *changes* in temperature which appear to be the controlling factor.

This suggestion, in spite of some contradictory evidence, is supported by a comparison of the following results, which moreover indicate that the more acute the change the greater the response. For example, in the "simple transference" experiments, detailed in Table XXXI, taking place in incubators 85 Wet and 85 Dry (Exp. (a) 30th Jan. 1911 and Exp. (b) 24th March, 1911) there is a break in the record of emergences between about the 25th day and the 70th day after the cocoons were transferred. The results of the same experiments are graphically expressed in Charts 7 and 8, in which the double period of emergence is clearly shown.

If I am not mistaken, this same feature may be observed in the other experiments (see, for example, Tables XXXI and XXXII and Chart 9) though not so clearly, and the suggestion is that we have here traces of an inherent discontinuity in the development of this species which in nature may favour a spring or autumn emergence of adults. It is an interesting question whether the individuals whose emergence is delayed by heat are the same as those whose emergence may be delayed by cold, or if the influence of extremes of temperature varies in different individuals. While there is no direct evidence

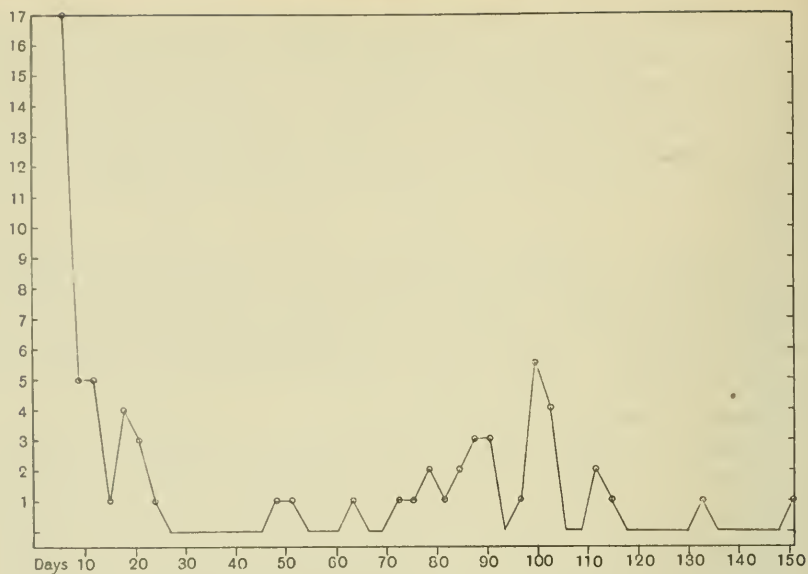


Chart 7. Emergence of *C. fasciatus* from cocoons in Incubators 85 Dry and 85 Wet (see Exps. Jan. and March 1911, Table XXXI, Nov. 1910 and April 1911, Table XXXII).

Temperature approximately 84° F.

Humidity average between .60 to .76.

Vertical numbers = units of emergence. Horizontal numbers = time in days (3 day units).

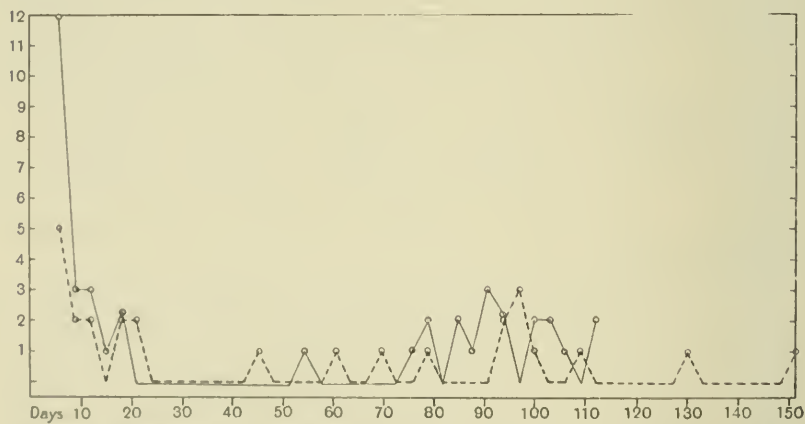


Chart 8. Emergence of *C. fasciatus* from cocoons in Incubators 85 Dry and 85 Wet (see Tables as Chart 7).

Continuous line, 85 Wet. Temperature 84° F. Humidity average .70 to .76.

Dotted line, 85 Dry. Temperature 81° F. Humidity average .60 to .63.

Vertical numbers = units of emergence. Horizontal numbers = time in days (3 day units).

on this point for this series, we may note the fact that the individuals reared under cool conditions in the cellar (7th October, 1910, Table XXVIII) during the autumn of 1910, assumed the cocoon stage during mid-winter, did not emerge during the spring but during the late summer and autumn of the following year. That is to say, that a proportion of them, after being reared under cool humid conditions, resisted the summer range of temperature with a mean average of above 60° F. for three months, and commenced their adult life on a falling temperature during the autumn of 1911.

A similar occurrence is also apparent in the Tables dealing with the cocoons spun in incubator 75 Wet, and then distributed to the cellar,

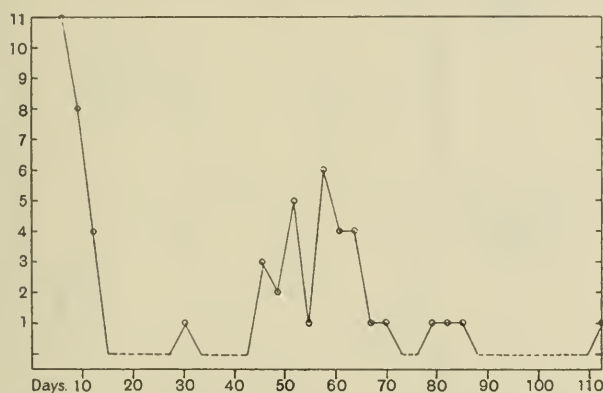


Chart 9. Emergence of *C. fasciatus* from cocoons in Incubator 75 Wet (see Exps. Jan., Feb., March 1911, Table XXXI, Nov. 1910, Table XXXII).

Temperature 74.6 to 75.5 F.

Humidity average from .76 to .84.

Vertical numbers=units of emergence. Horizontal numbers=time in days (3 day units).

laboratory cupboard and beehive (Table XXXI and Charts 10 to 12), and in the "complex transference" series, under date 8th November, 1910 (Table XXXII) spun in incubator 85 Wet, and then transferred to the cellar. No emergences at all took place between the 68th and 236th day after the cocoons were placed in the cellar. Perhaps the most striking example is afforded by an experiment in which cocoons were transferred from incubator 75 Wet to the beehive on the 23rd March, 1911 (see Table XXXI (b)). When the cocoons were put in, the mean temperature was only 42.9° F., yet some of the individuals resisted the high summer temperature of 1911, which reached a maximum average of 80° F. for the months of July and August, and emerged at much lower temperatures during the autumn.

Drought would appear to lengthen the average resting period in the cocoon at 85° F. and 92° F., and to shorten it at the lower temperature of 75° F. While there are some contradictions to be found in the tables, they do not counterbalance the general conclusion, and the massed evidence of the monthly series of tests—to be referred to immediately—is of the same tenor. The differences are not large, the duration of the cocoon period being on an average 25·3 days in 75 Wet, as against 23·8 days in 75 Dry, while at the higher

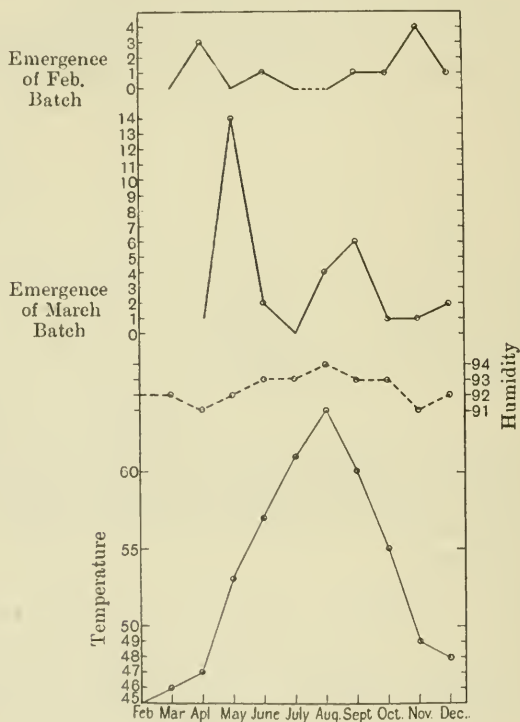


Chart 10. Influence of Temperature and Humidity upon the emergence of *C. fasciatus* from cocoons. CELLAR (see Exps. Feb. and March 1911, Table XXXI).

Two batches: one put in Feb., one put in March 1911.

Vertical numbers at top of chart give units of emergence (two series).

„ „ on Left, mean Temp. F°.

„ „ on Right, Humidity.

Horizontal divisions, time (monthly units).

Explanation. The two curves at the top of the diagram express the numbers of fleas emerging at monthly intervals of time. The dotted line represents the variation in humidity and the lower continuous line the variation of temperature during the period of experiment.

temperatures it is only 21.6 days in 85/93 Wet and 32.7 days in 85/93 Dry.

A very extensive series of experiments, of which only the summaries are included in Table XXXIII, were undertaken in order to investigate the existence of a seasonal fluctuation in the constitution of *C. fasciatus* which might influence the length of the resting period apart from the temperature. Full grown larvae were taken from the cages in large

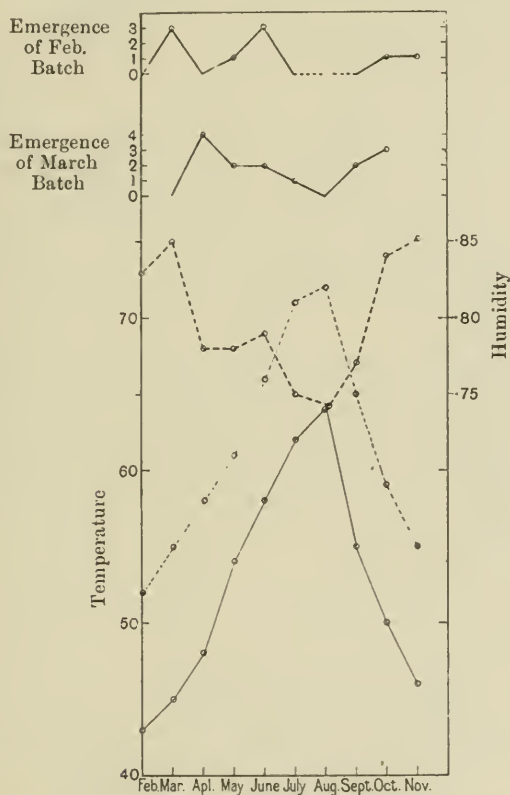


Chart 11. Influence of Temperature and Humidity upon the emergence of *C. fasciatus* from cocoons. LABORATORY CUPBOARD (see Exps. Feb. and March 1911, Table XXXI).

Two batches put in Feb. and March 1911.

Vertical numbers at top of chart give units of emergence (two series).

„ „ on Left, Temp. F°.

„ „ on Right, Humidity.

Horizontal divisions, time (monthly units).

Explanation as for Chart 10, except that temperature is represented by two curves (maxima and minima).

batches each month and placed in incubator 75° Wet, so that all the cocoons might be spun under conditions as nearly similar as possible. The cocoons were then divided into batches and distributed among the different incubators, cupboards, etc. as far as possible on identical dates.

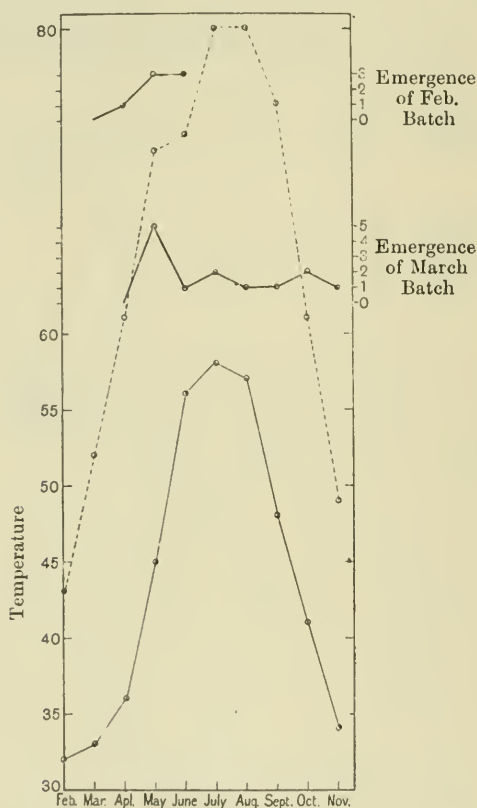


Chart 12. Influence of Temperature on emergence of *C. fasciatus* from cocoons (see Exps. Feb. and March 1911, Table XXXI).

Record of two batches in Beehive.

Vertical numbers on Right, units of emergence (two series). On Left, Temp. F.° scale. Horizontal divisions, time (monthly unit).

Explanation as for Chart 11.

Both in mortality and in tendency to "rest," there is no very clear indication of a seasonal change. The November cocoons certainly showed very definite intolerance of heat and drought. In the cool situations on the other hand there was a very marked tendency for them

to have prolonged resting periods, the average length of the cocoon period for this month being easily ahead of the others. The cocoons were also for the most part very hard.

Although the question cannot be regarded as settled, the results of the November series appear to negative the suggestion made on p. 539 that individuals which achieve lengthy periods of rest in response to cold were equally fitted for rest under hot conditions.

In short, the cocoon period of *C. fasciatus* is constitutionally a very varied one. It is possibly more correct to regard the species as having a certain percentage of individuals adapted for long resting periods in the face of extreme conditions of temperature, but susceptible to comparatively quick development in response to "warm" conditions (70° F.). There is also evidence which suggests that a "spring-autumn" emergence is favoured; individuals which have started to rest in response to low temperature resisting the early summer rise, continuing their rest in spite of a rising temperature and emerging during the autumn. It is even within the bounds of possibility that a second winter might be passed as larvae resting within the cocoons, since I have several instances of larvae resting for periods of over a year.

That a larger proportion of individuals achieve a lengthy rest under cool than hot conditions is perhaps a racial question; were we to experiment with a race of *C. fasciatus* which came from a warmer country than England, it is possible that the position might be reversed.

An experiment dealing with retarded emergence (Table XLIII) was made in the early spring of 1911. A large number of adult *C. fasciatus* were required to stock a new cage, and some 400 to 500 full-grown larvae were, therefore, taken from the cages, put into card jars with sand and shreds of B.S. rag and placed in incubators 75 Wet and 85 Wet. The fleas that emerged during March and April were used for the above purpose and did not form the subject of any special notice but the jars were examined every two or three days during May and onwards for "laggards."

The results are set forth in Table XLIII; this does not specifically belong to either the cocoon or larval series, it being uncertain when the cocoon period actually commenced. It is, however, probable that the greater portion of the time between the removal of the larvae from the cages and their emergence as fleas was passed within their cocoons. The Table is therefore included in this section and, as the numbers dealt with are considerable, they afford valuable testimony in support of the experiments, showing what a long period (up to 150 days) may

elapse, even under the influence of warm to hot conditions, before the full development of the adult is attained.

As regards the first batch, about 120 larvae were added to a jar contained in incubator 75 Wet on the 27th January, 1911; on the 4th March a further batch of larvae were added to this jar. The number of days stated are counted from the 4th March, although it is not improbable that some of the later records really belong to the January batch of larvae.

The cage referred to on p. 545 was stocked with some 200 to 250 fleas which may be termed the "forwards" of these batches. I am informed by Dr Boycott, in whose charge the cage remained for some two or three months, that three successive broods were observed in the cage with distinct intervals between them, during which the flea population fell to a minimum; very few individuals were seen in the intervals, but during the waves of emergence the cage swarmed with fleas.

X. cheopis (Tables XXXIV and XXXV). The effects of temperature are clear and unmistakable, the lengthening of the cocoon period being very marked at temperatures below 65° F.; situations with a mean temperature below this level as a rule give rise to a long rest within the cocoon. 65° F. would seem for this species to be equivalent to about 50° F. for *P. irritans* and 40 to 45° F. for *C. fuscatus*.

In the June 1911 series of experiments set forth in Table XXXV, the average duration of the cocoon stage in the incubators (15·5 days) is shorter than it was in the September experiments (21 days). As the other conditions in the two series were approximately the same, this difference suggests that some predisposing cause acted on the larvae before they were taken from the cages for the September experiment. Such an influence is probably not far to seek. Towards the end of September the temperature in the cages would be lower than during June, and it is also possible that the fall of temperature experienced by the larvae between the months of August and September may have provided the necessary stimulus for a longer resting period in the cocoon stage.

The degree of humidity does not seem to have had any marked influence on the length of rest in the cocoon. In the June series, there appears to be a slight advantage in the greater humidity of incubators, 85 Wet and 75 Wet, resulting in a shorter average length of cocoon period; this is, however, reversed in the September experiments when the incubators, 85 Dry and 75 Dry, give the shorter average. Corresponding contradictions are obtained when similar comparisons are made between other experiments in Table XXXV.

The results obtained are antagonistic to any supposition of an aestivating habit in the stock of *X. cheopis* used for these experiments.

In the September experiments of Table XXXV made in incubator 75 Wet, periods of 71 and 94 days in the cocoon were recorded. In the laboratory cupboard and cellar during the summer still longer times were taken for development. This must, I think, be regarded as a rest in response to moderate and cool conditions. This species is naturally adapted to a hot rather than a warm climate: a fact that is fully borne out by the susceptibility to cold or even cool conditions shown throughout these experiments.

At the high temperature of 93° F. the mortality of this species is not by any means prohibitive, provided the humidity is sufficient. Comparison of the results in the Wet and Dry incubators given under May, 1912, show this quite well (see Table XXXV).

The facts are, however, rather puzzling, with 93 Dry. They are more so in regard to 75 Dry, as the lowest readings of humidity were not by any means always accompanied by the highest mortality, and it is clear that there must be some disturbing factor. This may be due to the varied amount of ventilation that it is necessary to give to the incubators in order to keep the humidity as steady as possible. In December, where low figures for mortality were recorded, the ventilators were kept shut and all draught avoided. I fancy that the results are also affected by variation in the larvae, both in the matter of general vitality, which causes them to spin stronger or slighter cocoons (the flimsy ones being ill fitted to resist desiccation) and also in regard to the stage of preparation they have reached for the approaching metamorphosis before the cocoons are transferred to the dry incubators.

These results can only be taken to apply exactly for the race of English *X. cheopis* that were experimented with. It is quite possible that other races may possess greater powers of resistance to both heat and drought.

Ct. canis. The four Tables (XL (a), (b), (c) and (d)) dealing with this flea are not strictly comparable with each other. In the case of the earliest series under date 17th November 1910 (Table (b)) the figures recorded are definite as to dates of emergence but not as to the time of spinning; consequently the number of days calculated as the cocoon period are only approximate. With the July series (Table (c)), however, both series of dates are correct, but the number of cocoons used is approximate only; it was not possible to count them owing to their being spun together in masses. The experiment was the outcome of a chance

opportunity and we were too busily employed with definitely planned work to spare the time necessary to separate and count some 1300 cocoons used for this and the submergence trials. The figures given in the two April experiments (Table (a)) are also only approximate and may almost as correctly be supposed to deal with the larval as with the cocoon stage. The cocoons were left to emerge where the larvae span, with the consequence that there were emergences from cocoons that were not found. The experiment serves, however, to illustrate the effect of different conditions corresponding to incubator 75 Wet and the cellar in modifying the time of emergence, as both batches of larvae were placed under the test conditions on the same date.

It is to be noted that all the *Ct. canis* experiments are from stock naturally reared, not obtained from eggs laid under controlled conditions, nor, as with the rat fleas, bred from adults kept under exceptional, if favourable conditions. Of the November series (Table XL (b)) it may be remarked that the cellar experiment gives an approximately natural result; probably under out-of-door conditions in a kennel the emergence would have been some three weeks later. It is likely that the April series of larvae (Table XL (a)) are the progeny of adults that emerged in the spring about March, and the July larvae (Table XL (c) and (d)) follow from the adults reared from the April larvae. Such a basis suggests that *Ct. canis* produces about four or five broods a year according to the season, but it is probable that there would be overlapping of the fleas from each brood and a general emergence in August, provided warm weather prevailed. Some of the fleas emerging in autumn would probably survive the winter, but there would be few, if any, late autumn emergences under out-of-door conditions. The approximate unanimity displayed by this species in the incubators as regards the date of emergence of different individuals in one batch of cocoons (Table XL (c)) marks it off distinctly from the rat and human fleas and it seems not unlikely that the plagues of fleas said to occur suddenly, especially during late summer, are of this species.

Like *X. cheopis*, I can see no evidence of an aestivating habit from the recorded results in Table XL. A slender tail of laggards to each of the several batches makes considerably more show when the conditions are cool and moderate. Quite possibly many of these laggards are destroyed if high temperatures prevail.

Unfortunately no mortality figures can be given, as the cocoons were spun in masses, and at the period when this material was available there was no time to separate and count the cocoons accurately.

C. gallinae (Table XLI). The numbers available were too small to allow any but initiatory trials to be made and nothing very definite can be based on the results obtained. The chief items of interest would seem to be the low mortality in incubator 75 Dry, the frequent emergence of larvae from their cocoons followed by successful pupation and the fact that the mortality is so largely in the pupal stage. There is no evidence suggesting that this species may have an extended larval resting period within the cocoon.

L. musculi (Table XLII). A preliminary experiment, like that with *C. gallinae*, was undertaken; it is of some interest, but hardly affords basis for any final conclusions. This flea seems to be well adapted for the cool to moderate summer conditions of the cupboards, cellar and beehive, but not able to adjust itself readily to the high temperature of 93° F., although there is some evidence of its following *C. fasciatus* in attempting to meet unfavourable conditions both of heat and cold by resting. The recorded periods of rest are not comparable with those of *C. fasciatus*—this must be put down to want of opportunity, as it is quite uncertain how long the few examples that are now resting may take before emergence.

L. musculi would seem to be quite well adapted for conditions of low humidity in the cocoon stage—a fact that is significant, if we consider the habits and surroundings of its hosts in comparison with other flea hosts.

TABLE XXVIII. *Coccins* C. fasciatus. "Continuous" series, reared from eggs, each batch maintained during egg, larval, and cocoon stage in the same situation.

Date of commence- ment of experiment in the egg (or larval) stage, see note below.*	No. of cocoons found	Tempera- ture	Humidity (No. of cocoons)	Approximate duration of cocoon stage† Days	Incubator 75° Wet			Remarks
					Average number of days in cocoon	Mortality in cocoon stage	Food during larval period	
24 Sept. '10	7	74.4	.74	3 4	7 11	Nil	B.S. Rag	—
16 Oct. '10	1	74.1	.73	1 12	12	Nil	Rat faeces	—
30 "	8	74.2	.73	4 9	10	Nil	"	—
"				2 10				
"				1 11				
"				1 14				
"	13	74.2	.73	3 8	10	Nil	B.S. Rag	—
"				1 9				
"				6 10				
"				2 11				
"				1 14				
21 Dec. '10	8	74.6	.72	1 6	16	12 0/0	"	—
"				4 11				
"				1 13				
"				1 18				
14 Mar. '11	10	75.0	.79	1 6	11	?	Rat faeces chopped fine	5 cocoons opened 11 Aug. '11 found to be empty, probably false cocoons
"				1 10				
"				2 12				
"				1 17				
24 April '11	3	75.5	.88	3 15	15	Nil	Powdered blood	—

23 May '11	20	76.0	.86	5	9	13	Nil	Flea faeces
				8	12			
				6	14			
				1	44			
" "	7	76.0	.86	—	—	—	?	Rat faeces
" "	21	75.7	.85	6	9	12	5%	B.S. Rag
				7	12			
				7	14			
22 Sept. '10	5	84.3	.72	No records, all emerged		No records	Nil	B.S. Rag
15 Mar. '11	6	84.3	.71	1	4	7	Nil	Rat faeces chopped fine
				3	7			
				1	9			
				1	12			
8 July '11	29 Aug. 84.7 Sept. 84.8 Oct. 84.8		.81 .78 .78	3 6 3	24 25 32	44	20%	B.S. Rag
				2	43			
				2	47			
				1	69			
				1	71			
				3	75			
				1	103			
8 "	No cocoons	—	—	—	—	—	—	Rat faeces
13 "	6	84.9	.80	1	36	40	Nil	Flea faeces
				1	40			
				2	40			
				1	43			

* NOTE. Experiments in Table XXVIII (up to 14 Feb, 1911) are in direct continuity with those dealing with the egg stage given in Table II and (up to 25 June 1911) with larval stage experiments given in Table XVI.

† NOTE. It was not possible to fix the exact date of spinning; the days in cocoon (reckoned from the finding of the cocoons) are likely to be too few but their relation to each other should be correct.

Cocoons opened 23 Aug. '11 all empty
1 cocoon opened contained dried up larva

Remaining cocoons opened 1 Dec. '11 contained 1 dried up flea, 5 dried up larvae, 1 empty cocoon

Remaining cocoon opened and found empty, false cocoon

TABLE XXVIII.—Continued.

Laboratory Cupboard									
Date of commencement of experiment in the egg (or larval) stage	Number of cocoons found	Temperature	Humidity	Approximate duration of cocoon stage (No. of cocoons) Days		Average number of days in cocoon	Mortality in cocoon stage	Food during larval period B.S. Rag	Remarks
				1	102				
6 Aug. '10	1	60 (approximate only to 8 Nov.) max. min. Nov. 54.0 42.8 Dec. 56.5 47.8	.84 .84 .85	1	102	—	—		Cocoon opened 1 Jan. '11 and found to contain a living larva
12 "	4	60 (approximate only to 8 Nov.)	.84	1	23	26	Nil	"	—
				1	25				
				1	28				
Cellar									
31 July '10	1	max. min. 58.0 57.0 about	.93	1	27	—	Nil	"	—
4 Aug. '10	3	58.0 57.0 about	.93	1	21	26	Nil	"	—
				2	29				
14 "	1	58.0 57.0 about	.93	1	30	—	Nil	"	—
7 Oct. '10	12	Dec. 50.0 48.8	.93	2	203	236	—	"	1 opened 8 May '11 and found to contain a resting larva. Larva placed in small tube in 75 Wet. it died without undergoing change to pupa
	2	30/12/10 1911		1	219	(cocoon opened 21 Oct. '11 contained living flea)			
	1	2/ 1/11 Jan. 46.5 45.3	.92	2	224				
	6	9/ 1/11 Feb. 46.4 44.8	.92	2	269				
	1	19/ 1/11 Mar. 46.5 45.3	.91	1	285				
	2	28/ 1/11 April 48.5 46.6	.91	1					
		May 53.9 52.6	.92						
		June 58.1 56.7	.93						
		July 61.6 60.4	.93						
		Aug. 64.5 63.4	.94						
		Sept. 60.6 59.5	.93						
		Oct. 56.3 54.8	.92						

4 April '11	3	max. min. Nov. 50·7 48·9 Dec. 48·7 47·4 1912 Jan. 47·2 45·9 Feb. 45·8 44·6	— — — — —	— — — — —	— — — — —	Rat faeces 2 fleas emerged, 1 on 12 June, 1 on 27 July, but the cocoons they came from were not found	
18 June '11	32 21 July	see above	—	71	—	Flea faeces 1 cocoon opened contained dried up pupa	
25 "	27 21 July	see above	—	27	—	B.S. Rag 1 cocoon false, neither dried remains nor cast skin	
				19 26 31 39 41 47 54 61 70 81 97 105 121 131 138 194 207	2 3 3 4 3 2 2 1 1 1 1 2 1 1 1 2		
				13 19 26 25 31 41 54	1 4 9 4 6 1 1		

TABLE XXIX. *Cocoon* *C. fasciatus*. "Continuous" series reared from eggs.

Date of commencement of experiment in the egg (or larval stage) note below	Number of cocoons found	Temperature	Moistened	Incubator 75° Wet (moistened during the larval period)*			Mortality in cocoon stage	Food during larval period	Remarks
				Approximate duration of cocoon stage: (No. of cocoons) Days	Average number of days in cocoon	B.S. Ra ₂			
13 Jan. '11	9	73.7	Water	2 13 3 14 1 26 1 27 1 36	19	11 0/10	11 0/10		1 cocoon opened and found to contain a dead flea
28 Dec. '10	6	74.7	Urine	2 11 1 10	11	50 0/10	50 0/10		3 cocoons opened and dead fleas found in them
"	14	74.6	Water	2 13 2 18 1 21	16	64 0/10	64 0/10		9 cocoons opened contained 8 dead larvae, 1 dead flea
4 Mar. '11	2	74.6	Urine	2 13	13	Nil	Nil		only 2 cocoons found
"	1	74.6	Water	1 10	10	Nil	Nil		—
Incubator 75° Dry									
31 Jan. '11	3	75.7	Water daily	1 10-13	—	67 0/10	67 0/10		2 cocoons opened contained dead larvae
"	No cocoons	—	Urine daily	—	—	—	—		—
Incubator 85° Wet									
30 Dec. '10	7	85.3	Urine at intervals	5 13	—	29 0/10	29 0/10		2 cocoons opened contained dried up pupae
"	No cocoons	—	Water at intervals	—	—	—	—		—
24 Feb. '11	2	83.5	Urine 1 c.c. daily	1 3 1 6	5	Nil	Nil		—
"	4	84.2	Water 1 c.c. daily	1 16 1 20	18	50 0/10	50 0/10		2 cocoons opened contained dead larvae

					Incubator 85 Dry					
					1	13				
21 Feb. '11	2	84.0	Water 1 c.c. daily	—	1	14	Nil	B.S. Reg	—	—
"					—	—	—	—	—	—
15 May '11	No cocoons	—	Urine 1 c.c. daily	—	—	—	—	—	—	—
	6	June 83.9 July 84.2	Water .5 c.c. daily	—	1	13	34	"	3 cocoons opened	dried up
					1	34	50 %	"	larvae	
					1	55	—	—	—	—
6 "	13	May 84.0 June 83.9 July 88.5	Urine .5 c.c. daily	—	4	7	19	"	1	"
					1	15	8 %	"	"	"
					5	21	—	—	—	—
					1	39	—	—	—	—
					1	41	—	—	—	—
Warm Cupboard										
NOTE. No larvae of unmoistened experiments lived to spin cocoons in this cupboard.										
3 Feb. '11	1	63.5	Water daily	—	1	18	Nil	"	—	—
"	No cocoons	—	Urine daily	—	—	—	—	"	—	—
8 Mar. '11	10	63.1	Water 1 c.c. daily	—	2	12	19	"	—	—
					2	16	—	—	—	—
					1	18	—	—	—	—
					1	22	—	—	—	—
					3	24	Nil	"	—	—
					1	23	—	—	—	—
"	No cocoons	—	Urine 1 c.c. daily	—	—	—	—	"	—	—
5 May '11	7	66.8	Urine .5 c.c. daily	—	1	16	21	57 %	4 cocoons opened	dried up
					1	17	—	"	larvae	
					1	31	—	—	—	—
6 "	6	65.3	Water .5 c.c. daily	—	1	9	14	33 %	2	"
					1	14	—	—	—	—
					1	15	—	—	—	—
					1	20	—	—	—	—

* No larvae in case of experiments under unmoistened conditions in incubators 75 Dry and 85 Dry survived to spin cocoons.

+ Experiments in Table XXIX are in direct continuity with those dealing with the larval stage given in Table XVII, and (up to 8 March 1911) egg stage in Table I.

‡ The numbers given for the duration of the cocoon stage are likely to be too small but should bear a correct relation to one another; the date of spinning is uncertain, the date of finding the cocoon was alone recorded.

TABLE XXX. *Cocoons* C. fasciatus. "Continuous" series, contrasted experiments (compiled from Table XXVIII); larvae from same batch of eggs.

(a) Reared on the same food under different conditions of Temperature and Humidity.											
Date of commencement of experiment in the larval stage	Place reared from egg in	Food	Number of cocoons found	Temp	Humidity	Approximate duration of cocoon stage (No. of cocoons)	Days	Average number of days in cocoon	Mortality in cocoon stage Doubtful if any		
14 March '11	Incubator 75 Wet	Rat faeces crushed	10	75.0	.79	1	6	11			
						1	10				
						2	12				
						1	17				
						5 cocoons opened 11 August '11 and found empty, probably false cocoons					
15 March '11	Incubator 85 Wet	"	6	84.3	.71	1	4	7	Nil		
						3	7				
						1	9				
						1	12				
(b) Reared under the same conditions of Temperature and Humidity but on different food.											
Date of commencement of experiment in the egg stage 30 Oct. '10	Incubator 75 Wet	Reared from hatching on B.S. Rag	13	74.2	.73	3	8	10	"		
						1	9				
						6	10				
						2	11				
						1	14				
	"	Rat faeces	8	74.2	.73	4	9	10	"		
						2	10				
						1	11				
						1	11				
						1	14				

TABLE XXXI (a). *Cocoon C. fasciatus*. Full grown larvae taken from the breeding cages and allowed to spin in incubator 75 Wet (Temperature 74° F., Humidity 70); cocoons then placed in different situations.

Date on which the cocoons were placed under test conditions	No. of cocoons	Place	Temperature	Humidity	Dates of emergence of fleas	No. of days in cocoon	Average days	Remarks
30 Jan. '11	15	Incubator 85 Wet	84.0	.70	4 7 Feb. '11 1 10 " 2 13 " 1 20 " 1 16 April '11 1 21 " 1 12 May '11	8 11 14 21 76 81 102	32	23 March '11. Examined, 5 "soft" all empty, 3 "hard" contained resting larvae. 7 June '11. 5 "hard" and 1 "soft" opened, 1 contained living resting larva, remainder contained either dried up larvae or cast larval skins. Mortality doubtful
"	15	Incubator 85 Dry	84.1	.60	2 7 Feb. '11 2 8 " 1 20 " 1 23 " 1 24 " 1 6 April '11 1 27 " 1 3 July '11	8 9 21 24 25 66 87 154	41	19 Feb. '11. 7 empty cocoons opened all "soft" or tending in that direction, but not dimsy cocoons, the silk forming a continuous papery lining. One very little if at all stronger contained a resting larva. 7 "hard" cocoons unopened. Mortality doubtful 7 June '11. One of the hard cocoons opened found to contain a living pupa, the adult flea within the pupal envelope being nearly ready to emerge. Mortality doubtful
"	15	Incubator 75 Wet	75.5	.76	3 7 Feb. '11 1 10 " 1 21 March '11 2 24 " 1 2 April '11 1 3 "	8 11 50 53 62 63	35	23 March '11. 6 "soft" cocoons opened, 4 empty, 1 contained pupa with flea nearly ready to emerge, 1 a dead flea. 2 "hard" cocoons contained "white," that is recently developed, pupae 7 June '11. Remainder of cocoons opened contained either dried up larvae or cast larval skins. Mortality doubtful
"	15	Incubator 75 Dry	75.6	.49	3 7 Feb. '11 1 8 " 2 13 " 1 14 " 1 17 " 1 18 March '11 *1 25 " 1 27 " 1 31 "	8 9 14 15 18 47 54 56 60	26	13 March '11. 1 hard cocoon opened contained a resting larva which successfully pupated and an adult flea was reared on the 25th March, see column 6* Note the silk of this cocoon was hard and brown forming an almost horny lining to the cocoon June '11. Remaining cocoons opened contained dead larvae. Mortality 20%

TABLE XXXI (a)—Continued.

Date on which the cocoons were placed under best conditions	No. of cocoons	Place	Temperature max. min.	Humidity	Dates of emergence of fleas	No. of days in cocoon	Average days	Remarks
1 Feb. '11	13	Lab. cupboard	Feb. 52-8 43-6 Mar. 52-2 45-5 April 58-2 48-2 May 61-8 54-3 June 66-4 58-2 July 71-5 62-7 Aug. 72-0 64-1 Sept. 65-7 55-7 Oct. 59-0 50-9 Nov. 54-9 46-1	.83 .85 .78 .78 .79 .75 .74 .77 .84 .85	1 16 " 1 23 " 1 30 " 1 23 May '11 1 1 June '11 1 10 " 1 20 " * 1 3 Oct. '11 1 21 Nov. '11	43 50 57 80 89 98 108 244 293	118	23 March '11. 3 "soft" cocoons opened all empty, 2 slightly firmer contained 1 "white" pupa, 1 "resting" larva. 2 "hard" cocoons opened, 1 was empty, 1 contained resting larva. 3 Oct. '11. 2 cocoons opened, 1 was entirely empty, 1 contained a living flea, see column 6 21 Oct. '11. 1 cocoon opened contained a living pupa. Mortality doubtful
"	13	Cellar	Feb. 46-4 44-8 Mar. 46-5 45-3 April 48-5 46-6 May 53-9 52-6 June 58-1 56-7 July 61-6 60-4 Aug. 64-5 63-4 Sept. 60-6 59-5 Oct. 56-3 54-8 Nov. 50-7 48-9	.92 .91 .91 .92 .93 .93 .94 .93 .92 .91	1 8 April '11 1 13 " 1 15 " 1 20 June '11 1 26 Sept. '11 1 16 Oct. '11 2 9 Nov. '11 2 20 " 1 11 Dec. '11	66 71 73 139 237 257 281 292 313	209	Remaining cocoons opened and found empty, i.e. "false" cocoons. Mortality nil
4 Feb. '11	9	Beehive	Feb. 43-8 30-7 Mar. 52-5 33-3 April 61-7 36-8 May 72-8 45-2 June 73-0 56-5	Not recorded	1 22 April '11 2 2 May '11 1 30 " 2 6 June '11 1 10 "	77 87 115 122 126	103	25 Sept. '11. Two cocoons opened contained dried up larvae Mortality 22 %
15 Feb. '11	13	Warm cupboard	64-3	.53	1 1 March '11	14	—	19 March '11. 5 "soft" cocoons opened con- tained dead larvae, 7 "hard" cocoons opened also contained dead larvae <i>Note.</i> None of the cocoons were of equal strength and texture to the "hard" cocoons in the incubators Mortality 92 %

Special experiment with cocoons from one batch of larvae taken from the same date, 24 March 1911.

Date on which the cocoons were placed under test conditions	Number of cocoons	Place	Temperature	Humidity	Dates of emergence of flies	No. of days in cocoon	Average	Mortality	Remarks
24 March '11	15	Incubator 85° Wet	84.0	.76	5 2 April '11 2 3 " " 1 4 " " 1 6 " " 1 13 June '11 1 4 July '11	9 10 11 13 81 102	24	27%	Remaining cocoons opened 2 Oct. 1911 and found to contain dried up larvae
23 March '11	15	Incubator 85° Dry	84.0	.60	1 31 March '11 1 2 April '11 1 3 " " 1 7 " " 1 8 " " 1 15 " " 1 19 " " 1 9 June '11 3 5 July '11	9 11 12 15 16 23 27 78 104	46	27%	Remaining cocoons opened 23 Oct. 1911 contained 3 dried up fleas, 1 dried up larva
24 March '11	15	Incubator 75° Wet	75.4	.84	2 3 April '11 1 14 May '11 1 22 " " 1 23 " " 1 24 " " 1 30 " " 1 14 June '11 1 15 " " 1 19 " " 1 17 July '11	10 53 59 60 61 67 82 83 87 115	62	27%	Remaining cocoons opened 2 Oct. 1911 contained dried up larvae
"	15	Incubator 75° Dry (II)	75.7	.47	2 3 April '11 1 6 " "	10 13	11	80%	Remaining cocoons opened 28 Sept. 1911 contained dried up larvae
"	15	Incubator 75° Dry (I)	75.7	.47	2 3 " " 1 4 " " 1 6 " "	10 11 13	11	76%	Remaining cocoons opened 28 Sept. 1911 contained dried up larvae
"	15	Lab. cupboard	max. min. March 55.2 45.5 April 58.2 48.2 May 61.8 54.3 June 66.4 58.2 July 71.5 62.7 Aug. 72.0 64.1 Sept. 65.7 55.7 Oct. 59.0 50.9	.85 .78 .78 .79 .75 .74 .77 .84	4 29 " " 2 9 May '11 1 17 June '11 1 23 " " 1 27 July '11 1 20 Sept. '11 1 22 " " 1 9 Oct. '11 1 13 " " 1 23 " "	36 46 85 91 125 180 182 199 203 213	108	7%	1 cocoon opened contained dried up larva

TABLE XXXI (b)—Continued.

Date on which the cocoons were placed under test conditions	Number of cocoons	Place	Temperature max. min.	Humidity	Date of emergence of fleas	No. of days in cocoon	Average	Mortality	Remarks
24 March '11	15	Cellar I	March 46.0 44.7 April 48.5 46.6 May 53.9 52.6 June 58.1 56.7 July 61.6 60.4 Aug. 64.5 63.4 Sept. 60.6 59.5 Oct. 56.3 54.8 Nov. 50.7 48.9	-93 -91 -92 -93 -93 -94 -93 -92 -91	2 13 May '11 2 18 " 3 27 " 1 3 June '11 1 26 " 2 9 Aug. '11 2 21 " 1 14 Sept. '11 1 26 "	50 57 64 70 93 138 150 174 186	100	Nil	—
"	15	Cellar II	(as above)		1 13 May '11 4 18 " 2 27 " 1 11 Sept. '11 1 19 " 2 26 " 1 16 Oct. '11 1 15 Nov. '11 1 7 Dec. '11 1 25 "	50 57 64 171 179 186 206 236 258 276	140	Nil	—
23 March '11	15	Beehive	March 52.5 33.3 April 61.7 36.8 May 72.8 45.2 June 73.0 56.5 July 80.3 58.6 Aug. 80.2 57.6 Sept. 75.3 48.0	— — — — — —	1 9 May '11 2 13 " 1 18 " 1 30 " 1 14 June '11 1 14 July '11 1 24 " 1 2 Aug. '11 1 25 Sept. '11 1 30 " 1 3 Oct. 1 27 " 1 4 Nov.	47 51 58 68 83 113 123 132 186 191 194 218 226	124	6%	1 cocoon opened 30 Sept. '11 contained living flea Remaining cocoon contained dead flea
24 March '11	15	Warm cupboard	Dry March 61.7 April 63.0 May 62.9	-58 -57 -60	3 7 April '11 2 9 " 1 10 " 1 11 " 1 15 " 1 17 " 2 23 May '11 1 24 "	14 16 17 18 22 24 60 61	31	Nil	Remaining cocoons opened 30 Sept. '11 empty. ? "false" cocoon

TABLE XXXI (c). *Cocoon* C. fasciatus. Full grown larvae taken from the breeding cages and allowed to spin in Incubator 75 Wet (Temperature 73° F., Humidity 70); cocoons also maintained in Incubator 75 Wet.

Contrast between cocoons kept in card jar and tube.

Date on which the cocoons were placed under test conditions	Number of cocoons	Receptacle	Temperature	Humidity	Dates of emergence of fleas	Number of days in cocoon	Average	Mortality	Remarks
20 Feb. '11	15	Tube	74.6	.77	1 1 March '11	9	37	27 %	Remainder of cocoons opened 17 Aug. '11, found 1 dried up pupa, 3 dried up larvae
					2 2 "	10			
					2 4 "	12			
					2 13 April '11	52			
					1 15 "	54			
					1 21 "	60			
					1 24 "	63			
					1 2 May '11	71			
					2 28 Feb. '11	8			
"	15	Jar	74.6	.77	1 1 March '11	9	41	20 %	Remainder of cocoons opened 18 Aug. '11, found 1 dried up flea, 2 dried up larvae
					1 4 "	12			
					3 10 April '11	49			
					1 15 "	54			
					1 20 "	59			
					1 21 "	60			
					1 24 "	63			
					1 30 "	69			

TABLE XXXII.—Continued. *Cocoons C. fasciatus.*

No. of larvae	Place in which the larvae formed their cocoons and dates on which they were found and transferred	Temp.		Humidity	Place to which the cocoons were transferred and dates of emergence of fleas	Temp.	Humidity	Estimated no. of days in cocoon	Av.	Mortality
		max.	min.							
26	Beehive 1 19 April '11 (25 larvae died)	69.6	33.3	—	Incubator 85° Wet	83.0 (No emergence from cocoon)	.76	—	—	—
Unre- corded	Incubator 85° Wet 22 6 June '11	76.4		.93	Cellar 3 5 July '11 1 12 " 1 27 " 1 1 Aug. '11 2 9 " 1 11 " 3 21 " 1 11 Sept. '11 1 14 " 2 19 " 1 26 " 1 16 Oct. '11 1 1 Nov. '11 1 15 " 1 20 " 1 7 Dec. '11	1911 max. min. June 58.1 56.7 July 61.6 60.4 Aug. 61.5 63.4 Sept. 60.6 59.5 Oct. 56.3 54.8 Nov. 50.7 48.9 Dec. 48.7 47.4 1912 Jan. 47.2 45.9 Feb. 45.8 44.6 Mar. 49.1 48.1 Apr. 50.3 49.1	.93 .93 .94 .93 .92 .91 .92 3 " 76 1 " 97 1 " 100 2 " 105 1 " 112 1 " 132 1 " 148 1 " 162 1 " 167 1 " 184	88	Nil	
Unre- corded	Incubator 75° Wet 14 6 June '11 17 8 "	1911 June 75.8		.86	Cellar 3 5 July '11 4 12 " 3 11 Aug. '11 2 21 " 1 11 Sept. '11 3 14 " 4 19 " 1 26 " 2 5 Oct. '11 1 16 " 1 1 Nov. '11 1 9 " 1 26 April '12	(as above)		3 took 29 4 " 36 3 " 66 2 " 76 1 " 97 2 " 98 1 " 100 4 " 103 1 " 110 2 " 119 1 " 130 1 " 146 1 " 154 1 " 323	91	about 3%

(Remaining cocoons opened contained 1 dead flea, 3 false cocoons)

(Remaining cocoons opened contained 1 dead flea, 3 false cocoons)

TABLE XXXIII. Cocoons of *C. fasciatus*, mounting test. June 1911 to February 1912. Full grown larvae taken from the breeding cages and allowed to spin in Incubator 75° Wet; cocoons then removed to various situations.

Incubator 75° Wet.									
Date on which the cocoons were placed under test conditions	Number of cocoons	Temperature	Humidity	Min. days in cocoon	Max. days in cocoon	Average number of days in cocoon	Number of fleas emerging	Mortality	Remarks
17 June '11	26	June 75·8	·86	10	59	16	26	Nil	—
11 July '11	22	July 76·3	·85	13	72	42	21	Nil	1 "false" cocoon
15 Aug. '11	22	Aug. 75·0	·80	13	59	24	21	Nil	"
20 Sept. '11	22	Sept. 74·8	·76	12	54	27	18	10 0/10	as larvae, 2 "false" cocoons
20 Oct. '11	22	Oct. 74·4	·73	11	57	25	17	14 0/10	as fleas, 2 "false" cocoons
15 Nov. '11	22	Nov. 75·0	·69	10	82	31	22	Nil	—
10 Dec. '11	22	Dec. 75·8	·65	9	64	15	18	18 0/10	9 0/10 as larvae, 9 0/10 as fleas
5 Jan. '12	22	1912		15	58	22	21	Nil	1 "false" cocoon
11 Feb. '12	22	Jan. 75·5	·71	8	79	27	20	5 0/10	as flea, 1 "false" cocoon
		Feb. 75·0	·76						
		Mar. 75·1	·79						
		April 75·8	·79						
		May 75·6	·86						
		June 74·9	·83						
		July 75·0	·86						
		Aug. 74·3	·81						
Incubator 75° Dry.									
17 June '11	22	June 75·2	·53	9	27	15	20	10 0/10	as larvae
11 July '11	22	July 76·3	·57	13	70	31	4	72 0/10	" 2 "false" cocoons
14 Aug. '11	22	Aug. 75·7	·59	11	23	14	22	Nil	—
18 Sept. '11	22	Sept. 74·9	·54	10	45	18	14	27 0/10	22 0/10 as larvae, 5 0/10 as fleas, 2 "false" cocoons
20 Oct. '11	22	Oct. 74·6	·52	11	60	24	19	Nil	3 "false" cocoons
16 Nov. '11	22	Nov. 74·4	·54	11	68	28	6	72 0/10	as larvae
12 Dec. '11	22	Dec. 74·7	·51	12	70	24	20	9 0/10	"
4 Jan. '12	22	1912		8	128*	34	20	9 0/10	"
11 Feb. '12	22	Jan. 75·2	·50	11	86	36	17	14 0/10	"
		Feb. 73·5	·56						
		Mar. 73·2	·56						
		April 74·1	·56						
		May 74·7	·59						
		June 75·8	·54						
		July 76·2	·64						
		Aug. 75·0	·56						

* A cocoon opened on the 11th May contained a living flea.

TABLE XXXIII.—Continued.

Date on which the cocoons were placed under test conditions	No. of cocoons	Temperature	Humidity	Min. days in cocoon	Max. days in cocoon	Average number of days	Incubator 85/93 Dry.
							Number of fleas emerging
							Mortality
							Remarks
June 83-9	28	'60	8	117	32	16	4 0/10 fleas, 39 0/10 larvae
July 84-3	22	'64	14	92	48	14	31 0/10 larvae, 5 0/10 fleas
Aug. 83-8	22	'63	14	66	23	15	1 dead flea, 1 dead larva, 5 empty cocoons, doubtful if "false" cocoons or larval re-
Sept. 84-4	22	'59					mains past recognition
Oct. 84-7	22	'60	11	40	17	17	5 0/10 larvae, 5 0/10 fleas, 3 "false" cocoons
Nov. 92-9	22	'56	18	24	21	13	10 0/10 fleas, 20 0/10 larvae, 3 "false" cocoons, 2 cocoons opened 16 March contained living
Dec. 92-8	22	'57					but somewhat shrunken larvae which sub-
Jan. 93-2	22	'56					sequently died
Feb. 93-0	22	'60	1 flea emerged in 7 days	7		1	2 larvae emerged from cocoons 20 Nov. and died; cocoons opened 16 March contained
Mar. 93-1	22	'61					11 dead and dried larvae, 1 doubtful if dead
Apr. 93-7	22	'58					and as it still retained its natural colour and
May 93-3	22	'55					appearance but did not move, 2 dead fleas,
June 93-0	22	'57					remainder "false" cocoons
July 93-0	22	'54	10	118	77	11	cocoons examined 16 March '12; 4 opened contained 3 resting larvae*, 1 empty; the
Aug. 93-0	22						remaining cocoons and 3 extracted larvae were transferred to Incubator 75 Wet and
							6 fleas emerged; on 31 May 1912 a cocoon opened contained a dead flea
							cocoons opened 28 June contained 8 dried up larvae, 5 dried up pupae, 9 dried up fleas
13 Feb. '12	22						as larvae
							5 0/10 as larvae, 10 0/10 as fleas
							as fleas
							as pupae
							5 0/10 as fleas, 5 0/10 as larvae; 2 cocoons opened
							11 May '12 contained resting larvae (204
							days); they were transferred to Incubator
							75 Wet with 1 unopened cocoon; the resting
							larvae died, the cocoon was opened 12 June
							and found to contain a dead larva
							5 0/10 as fleas, 5 0/10 as pupae, 59 0/10 as larvae;
							23 0/10 as fleas, 9 0/10 as pupae, 18 0/10 as larvae;
							2 resting larvae taken from their cocoons
							after 203 days were transferred to Incubator
19 June '11	28	'80	8	74	19	25	
19 July '11	22	'83	12	105	42	19	
14 Aug. '11	22	'81	7	42	28	17	
20 Sept. '11	22	'77	7	30	13	15	
20 Oct. '11	22	'78	13	31	15	18	
		'73					
		(to 23rd	85-2				
		Nov. 1 to 30th	75-0				
		Dec. 92-4					
		1912					
		Jan. 92-6					
		Feb. 92-4					
		Mar. 92-3					
		April 93-1					
15 Nov. '11	22	'66	10	28	14	6	69 0/10
12 Dec. '11	22	'68	7	25	14	9	23 0/10
		'67					
		'69					
		'66					
		'68					
		'67					
		'69					

Date	Temp.	Humidity	Wind	Clouds	Remarks
11 Feb. '12	22	68%	4	—	fresh and natural in appearance as though but recently dead
17 June '11	22	Nil	—	—	23% as fleas, 4% as pupae, 41% as larvae; 3 of these larvae though shrunken were still fresh and natural in appearance as though but recently dead
10 July '11	22	72%	54	—	4% as fleas, 68% as larvae
14 Aug. '11	22	27%	25	—	"
16 Sept. '11	22	5%	33	—	"
23 Oct. '11	22	14%	31	—	"
13 Nov. '11	22	64%	43	—	5% as fleas, 59% as larvae, 1 "false" cocoon as larvae, 2 "false" cocoons
11 Dec. '11	22	9%	12	—	"
4 Jan. '12	22	18%	26	—	"
19 Feb. '12	22	14%	25	—	1 "false" cocoon
15 June '11	22	Nil	—	—	as larvae
10 July '11	22	Nil	—	—	1 larva emerged from cocoon and died; 1 dead flea found in cocoon; 1 resting larva taken from cocoon after 357 days
11 Aug. '11	22	9%	37	—	as larvae
15 Sept. '11	22	9%	73	—	as larvae; 1 resting larva taken from cocoon
20 Oct. '11	22	5%	64	—	as larvae
13 Nov. '11	22	5%	261	—	as fleas, died in cocoons; 1 living pupa taken from cocoon after 270 days
11 Dec. '11	22	9%	101	—	as flea, died in cocoon; 1 living pupa taken from cocoon after 246 days, 1 resting larva taken from cocoon after 246 days
4 Jan. '12	22	5%	59	—	1 living flea taken from cocoon after 198 days, 1 living pupa taken from cocoon after 198 days
21 Feb. '12	22	Nil	67	—	1 living pupa taken from cocoon after 198 days

* One of the extracted larvae pupated on the 27th March '12 and the NOTE. Mortality "as fleas" implies that death occurred in cocoons.

TABLE XXXIII.—Continued.

Beehive.

Date on which the cocoons were placed under test conditions	No. of cocoons	Temperature max.	Humidity	Min. days in cocoon	Max. days in cocoon	Average number of days in cocoon	Mortality	Remarks
17 June '11	22	73.0	—	23	97	37	Nil	4 "false" cocoons
10 July '11	22	80.3	—	14	82	37	30%	25% died as larvae, 5% died as pupae, 2 "false" cocoons
11 Aug. '11	22	80.2	—	17	45	34	30%	died as larvae, 2 "false" cocoons
15 Sept. '11	22	75.3	—	28	75	40	Nil	1 resting larva found on opening cocoons on 22 June '12 living after 272 days, it died without pupating
23 Oct. '11	22	61.9	—	71	319	125	Nil	1 resting larva taken from cocoon after 255 days, 1 living flea taken from cocoon after 319 days
13 Nov. '11	22	43.6	—	99	324	196	5%	as flea; 1 living larva taken from cocoon after 234 days, 1 living larva taken from cocoon after 298 days, 1 living pupa taken from cocoon after 298 days, 1 living flea taken from cocoon after 298 days
11 Dec. '11	22	67.9	—	33	180	98	Nil	—
4 Jan. '12	22	73.4	—	86	246	117	23%	9% larvae, 5% pupae, 9% fleas; 1 living flea taken from cocoon on 6 Sept., 1 living pupa taken from cocoon on 6 Sept.
24 Feb. '12	22	64.9	—	45	195	92	Nil	1 living larva taken from cocoon after 517 days
15 June '11	22	58.1	.93	27	109	49	Nil	—
10 July '11	22	61.6	.93	22	390	78	Nil	1 resting larva found in cocoon after 348 days, 1 resting larva found in cocoon after 424 days
11 Aug. '11	22	61.4	.91	17	122	33	4%	as flea
15 Sept. '11	22	60.6	.93	31	329	88	10%	5% as larva, 5% as pupae; 1 resting larva from very hard cocoon after 281 days, 1 living pupa after 358 days
23 Oct. '11	22	56.3	.92	35	119	57	5%	as larva; 1 resting larva from cocoon after 243 days, 1 living pupa from cocoon after 320 days
13 Nov. '11	22	47.2	.92	53	450	296	9%	2 dead larvae, 1 living larva taken from cocoon after 222 days
11 Dec. '11	22	45.8	.93	35	303	131	5%	1 dead larva, 2 living fleas taken from cocoon after 271 days
4 Jan. '12	22	54.2	.93	63	247	92	Nil	1 living flea extracted from cocoon after 247 days
19 Feb. '12	22	56.4	.93	37	245	83	Nil	2 living pupae taken from cocoons after 201 days
22	22	57.5	.93					

Cellar.

cocoon stage in the same station.

Cellar.

Date of commencement of experiment in larval stage	Number of cocoons found	Temperature		Humidity	Approximate duration of cocoon stage	Average number of days in cocoon	Mortality in cocoon stage	Food	Remarks
6 June '11	2	max.	min.		No. of cocoons	Days			
	13	Aug. 64.5	63.4	.94	1	147	—	B.S. Rag	Second cocoon opened contained dried up flea
3 July '11	14	Sept. 60.6	59.5	.93	1	41	90	Flea faeces	Remaining cocoons opened 3 July '12 contained 4 dried up fleas, 1 "false" cocoon
		Oct. 56.3	54.8	.92	3	68			
		Nov. 50.7	48.9	.91	1	77			
		Dec. 48.7	47.4	.92	1	96			
					1	114			
					1	191			
					3	34	69	Rat faeces crushed	Remaining cocoons opened 3 July '12 contained 1 dried up flea, 1 dried up larva, 1 "false" cocoon
					2	41			
					1	50			
					2	91			
					1	96			
					1	114			
					1	128			
Incubator 85 Wet.									
11 Aug. '11	12	84.8		.78	7	11	13	B.S. Rag	—
					4	15			
					1	18			
	7	84.8		.78	1	7	12	Flea faeces	—
					3	11			
					3	15			
4 Sept. '11	22	84.7		.78	1	9	13	B.S. Rag	2 cocoons opened and found to be empty ("false" cocoons)
					2	11			
					1	12			
					9	14			
					5	15			
					2	18			
Incubator 75 Wet.									
	21	74.7		.70	1	16	22		2 cocoons opened after 46 days contained living fleas
					4	17		"	
					7	19			
					5	20			
					2	24			
					2	46			

* Experiments in Table XXXIV are in direct continuity with those dealing with the larval series in Table XX.

TABLE XXXV. *Cocoons X. Cheopis. Full grown larvae taken from the cages and allowed to spin in Incubator 75° Wet (Temp. 76° F., Humidity .84); cocoons then placed in different situations.*

Note. Experiments on the same date are from the same batch of cocoons.

Date on which the cocoons were placed under test conditions	No. of cocoons	Place	Temperature max. min.	Humidity	Date of emergence of fleas	Duration of cocoon stage No. of cocoons	Aver. days	Mortality per cent.	Remarks
16 June '11	14	Cellar	June 58-1 56-7 July 61-6 60-4 Aug. 64-5 63-4 Sept. 60-6 59-5 Oct. 56-3 54-8 Nov. 50-7 48-9 Dec. 48-7 47-4	.93 .93 .94 .93 .92 .91 .92	1 on 16 Aug. 3 " 21 " 1 " 5 Sept. 1 " 14 " 1 " 20 " 1 " 11 Nov. 1 " 2 Dec.	1 3 1 1 1 1 1	61 66 81 90 96 148 169	35 35	Cocoons opened, 5 dead fleas
"	14	Lab. Cupboard	June 66-4 58-2 July 71-5 62-7 Aug. 72-0 61-1 Sept. 65-7 55-7	.79 .75 .74 .77	1 " 21 July 1 " 27 " 1 " 2 Aug. 1 " 9 " 2 " 16 " 4 " 30 " 1 " 22 Sept.	1 1 1 1 2 4 1	35 41 47 54 71 75 98	21 21	3 cocoons opened 21 Oct. contained 1 larva died in pupation, 1 dried up flea, 1 dried up pupa
19 "	14	Warm Cupboard	69-8	.66	2 " 10 July 1 " 13 " 2 " 15 " 3 " 17 " 3 " 26 " 1 " 31 " 1 " 14 " 2 " 18 " 6 " 24 " 3 " 29 " 1 " 2 Aug.	2 1 2 3 3 1 1 2 6 3 1	21 24 26 28 37 42	14	2 cocoons opened 24 Oct. contained 1 dried up flea, 1 dried up larva
"	13	Beehive	June 73-0 56-5 July 80-3 58-6 Aug. 80-2 57-6	—	1 " 14 " 2 " 18 " 6 " 24 " 3 " 29 " 1 " 2 Aug.	1 2 6 3 1	25 29 35 40 44	Nil	—
21 "	16	Incubator 75° Dry	76	.57	2 " 5 July 2 " 10 " 2 " 11 " 1 " 13 " 1 " 8 Aug.	2 2 2 1 1	14 19 20 22 48	50	8 cocoons opened 23 Oct. contained 2 dried up pupae, 6 dried up larvae
26 "	14	" 75° Wet	76-1	.86	7 " 10 July 2 " 11 " 2 " 13 " 1 " 17 "	7 2 2 1	14 15 17 21	Nil	2 cocoons opened 2 Oct. and found to be empty ("false" cocoons)

TABLE XXXV.—Continued.

Date on which the cocoons were placed under test conditions	No. of cocoons	Place	Temperature	Humidity	Date of emergence of fleas	Duration of cocoen stage No. of cocoons	Aver. days	Mortality per cent.	Remarks
23 Sept. '11	22	Incubator 85° Wet	85.1	.77	4 on 7 Oct. 3 " 10 " " 1 " 14 " " 1 " 2 " " 7 " 5 " " 3 " 19 " " 4 " 14 " " 4 " 19 " " 2 " 23 " " 2 " 31 " " 2 " 14 " " 7 " 19 " " 2 " 24 " " 1 " 5 Dec. 1 " 28 " " 6 " 11 " " 2 " 13 " " 6 " 16 " " 1 " 19 " " 5 " 13 " " 2 " 16 " " 7 " 19 " " 1 " 22 " " 2 " 16 " " 5 " 19 " " 1 " 22 " " 7 " 23 " " 1 " 24 " " 2 " 25 " " 5 " 16 " " 3 " 19 " " 1 " 22 " " 2 " 25 " "	4 14 17 21 9 12 26 19 24 28 36 19 24 29 71 94 10 12 15 18 9 13 15 18 17 15 18 21 21 12 15 18 			

7	"	22	Beehive						—	None emerged	—	—	—	100	2 larvae came out of their cocoons and died; cocoons opened 4 July '12 contained 18 dried up larvae, 2 "false" cocoons
"	"	22	Cellar						.92	None emerged	—	—	—	100	Cocoons opened 5 July '12 contained 19 dried up larvae, 3 "false" cocoons
"	"	22	Lab. Cupboard						.87	None emerged	—	—	—	100	2 larvae came out of their cocoons and died; cocoons opened 3 July '12, 1 dried up pupa, 19 dried up larvae
6 April '12		22	Incubator 93 Dry						.63	2 on 15 April 1 " 20 " " 2 " 22 " "	2 1 2	9 14 16	13	27	Remaining cocoons opened 6 Sept., 2 dead fleas, 4 dead larvae, 15 empty (11 "false" cocoons)
9	"	23	" 93 Wet						.70	3 " 18 " " 4 " 20 " " 7 " 23 " " 1 " 25 " "	3 4 7 1	9 20 14 16	12	30	Remaining cocoons opened 4 Sept., 2 dead fleas, 1 dead pupa, 4 dead larvae (1 "false" cocoon)
12	"	22	" 75 "						.79	11 " 27 " " 2 " 30 " " 4 " 6 May 1 " 10 " " 1 " 28 June	11 2 4 1 1	15 18 24 28 77	21	5	Remaining cocoons opened 5 Sept., 1 dead flea (2 "false" cocoons)
13	"	22	" 75 Dry						.56 .59	None emerged	—	—	—	100	4 larvae came out of cocoons and died; remaining cocoons opened 5 Sept., 17 dead larvae (1 "false" cocoon)
"	"	22	Cellar						.93	None emerged	—	—	—	100	Cocoons opened 5 July '12, 19 dried up larvae, 3 "false" cocoons
15	"	22	Lab. Cupboard						.83 .87	1 on 25 May	1	40	—	95	Remaining cocoons opened 6 July '12, 16 dried up larvae, 4 dried up pupae, 1 dried up flea
"	"	22	Beehive						—	None emerged	—	—	—	100	Cocoons opened 4 July '12, 21 dried up larvae, 1 "false" cocoon
"	"	22	Warm Cupboard						.64 .70	2 on 20 May 1 " 31 " "	2 1	35 46	—	77	Cocoons opened 4 July '12, 4 dried up pupae, 12 dried up larvae, 3 "false" cocoons
10 May '12		50	Incubator 75 Wet						.86 .83 .86	13 " 24 " " 19 " 28 " " 10 " 31 " " 5 " 13 June 1 " 26 " " 2 " 8 July	13 19 10 5 1 2	14 18 21 34 47 58	21	Nil	—
"	"	50	" 75 Dry						.59 .54	7 " 28 May 8 " 31 " " 5 " 3 June 2 " 6 " "	7 8 5 2	18 21 24 27	21	50	1 larva emerged from its cocoon and died; remaining cocoons opened 5 Sept., 23 dead larvae, 1 dead pupa (3 "false" cocoons)

"	"	50	"	93 Dry	May 93-3 June 93-0	.58 .55	None emerged	—	—	100	11 larvae emerged from cocoons and died; cocoons opened 5 Sept., 13 dried up fleas, 26 dried up pupae
"	"	50	"	75 Wet	May 75-6 June 74-9 July 75-0	.86 .83 .86	7 on 6 June 6 " 8 " 12 " 12 " 17 " 17 " 2 " 21 " 2 " 24 " 2 " 24 " 2 " 24 July	7 6 12 17 2 2 2 2	13 15 19 24 28 31 61	2	Cocoons opened 5 Sept., 1 dead flea (1 "false" cocoon)
28 May '12	"	50	"	75 Dry	75-8	.57	1 " 8 June 12 " 12 " 11 " 17 " 6 " 21 "	1 12 11 6	11 15 20 24	18	3 larvae emerged from cocoons and died; remaining cocoons opened 5 Sept., 2 dead fleas, 1 dead pupa, 10 dead larvae (4 "false" cocoons)
"	"	50	Cellar		max. min. June 56-4 55-6 July 60-1 59-3 Aug. 57-5 56-5 Sept. 56-0 54-3	.93 .93 .93 .92	3 " 15 July 1 " 19 " 1 " 26 " 2 " 3 Aug. 3 " 9 " 5 " 13 " 2 " 24 " 2 " 30 " *4 " 7 Sept.	3 1 1 2 3 5 2 2 4	48 52 59 67 73 77 88 94 102	26	Remaining cocoons opened 7 Sept., 10 dead pupae, 13 dead larvae * 4 living fleas (4 "false" cocoons)
"	"	50	Lab. Cupboard		June 64-6 58-3 July 68-5 62-1 Aug. 63-3 56-3 Sept. 61-5 53-0	.81 .79 .84 .86	4 " 3 July 2 " 15 " 3 " 20 " 8 " 27 " 2 " 2 Aug. 5 " 9 " 1 " 14 " +3 " 6 Sept.	4 2 3 8 2 5 1 3	36 48 53 60 66 73 78 101	62 (?) 18	Cocoons opened 6 Sept., 8 dead larvae, 1 dead pupa + 3 living fleas, remainder of cocoons lost through an accident
"	"	50	Beehive		June 67-7 49-9 July 73-4 55-4 Aug. 64-9 45-6	—	1 " 27 June 1 " 4 July 4 " 15 " 14 " 20 " 5 " 26 " 1 " 2 Aug. 1 " 11 " 1 " 24 "	1 1 4 14 5 1 1 1	30 37 48 53 59 66 75 88	54	Remaining cocoons opened 6 Sept., 12 dead larvae, 4 dead fleas, 3 dead pupae (3 "false" cocoons)
"	"	50	Warm Cupboard		June 65-0 July 67-3	.73 .71	17 " 21 June 12 " 27 " 4 " 2 July 1 " 12 " 2 " 20 " 1 " 26 "	17 12 4 1 2 1	24 30 35 45 55 61	33	2 larvae emerged from their cocoons and died; cocoons opened 5 Sept., 6 dead larvae, 2 dead fleas (3 "false" cocoons)

TABLE XXXV.—Continued.

Date on which the cocoons were placed under test conditions	No. of cocoons	Place	Temperature	Humidity	Dates of emergence of fleas	No. of cocoons	Duration of cocoon stage, Days	Aver. days	Mortality percent.	Remarks
3 June '12	50	Incubator 75 Wet	75.1	.83	1 on 12 June 23 " 17 " 9 " 21 " 13 " 24 "	1 9 9 21	1 14 18 21	17	Nil	Remaining cocoons opened 5 Sept. empty ("false" cocoons)
" "	25	"	75.8	.59	1 " 28 " 16 " 17 " 10 " 21 " 2 " 25 "	1 16 9 2	1 14 18 21	16	44	6 larvae emerged from their cocoons and died; cocoons opened 5 Sept., 13 dead larvae, 1 dead pupa, 2 dead fleas
3 "	50	"	94.2	.78	3 " 12 " 3 " 17 " 4 " 21 " 1 " 24 "	3 3 4 1	3 14 18 21	14	52	Remaining cocoons opened 4 Sept., 2 dead fleas, 15 dead pupae, 9 dead larvae, 13 "false" cocoons (?)
" "	50	"	93.0	.54	4 " 12 " 2 " 17 "	4 2	9 14	11	82	7 larvae emerged from their cocoons and died: remaining cocoons opened 5 Sept., 4 dead fleas, 19 dead pupae, 11 dead larvae, 3 "false" cocoons (?)
6 "	42	Cellar	max. min. June 56.4 55.6 July 60.1 59.3 Aug. 57.5 56.5 Sept. 56.0 54.3	.93 .93 .93 .92	4 " 26 July 6 " 9 Aug. 3 " 13 " *2 " 7 Sept. 1 " 14 Oct. *2 " 14 "	4 6 3 2 3 3	50 64 68 93 130	76	Doubtful	23 cocoons opened 7 Sept., 17 empty, 2 dead fleas, 2 dead pupae, 2 live fleas*, 19 cocoons opened 14 Oct., 13 empty, 2 dead fleas, 2 dead pupae, 1 dead larva, 2 live fleas* ("false") cocoons unrecorded, presumably 15)
12 July '12	50	Incubator 75 Wet	July 75.0 Aug. 74.3	.86 .81	20 " 24 July 20 " 28 " 2 " 5 Aug.	20 20 2	12 16 23	14	14	Cocoons opened 5 Sept., 5 dead larvae, 2 dead pupae (1 "false" cocoon)
" "	50	"	July 76.2 Aug. 75.0	.64 .56	8 " 24 July 8 " 25 " 14 " 28 " 7 " 31 " 1 " 3 Aug.	8 8 14 7 1	12 13 16 19 22	15	14	Remaining cocoons opened 5 Sept., 3 dead fleas, 3 dead pupae, 1 dead larva (5 "false" cocoons)
" "	50	"	95.3	.79	5 " 24 July	—	—	12	76	Cocoons opened 4 Sept., 13 dried up fleas, 16 dried up pupae, 9 dried up larvae (?) 7 "false" cocoons)
" "	50	"	93.0	.58	16 " 20 " 1 " 24 " 1 " 28 " 1 " 31 "	16 1 1 1	8 12 16 19	9	60	Cocoons opened 5 Sept., 4 dead fleas, 12 dead pupae, 14 dead larvae (1 "false" cocoon)

TABLE XXXVI. *Cocoon* *P. irritans*. "Continuous" * series, reared from eggs, each batch maintained during egg-larval and cocoon stage in the same situation.

Inenbator 75 Wet.

Date of commence- ment of experiment in the egg for larval stage	No. of cocoon- s found	Temp.	Humidity	Approximate duration of cocoon stage Days	Average duration of cocoon days in cocoon	Mortality in cocoon stage	Food	Remarks
13 July '10	2	75.0	Unrecorded	1 8 1 13	10	Nil	Rat faeces	—
19 "	3	75.0	.67	1 8 1 11	9	33%	Dead flies	—
11 Aug. '10	4	75.0	.60	4 12	—	Nil	B.S. Rag	—
1 Oct. '10	1	74.4	.73	1 15	—	Nil	B.S. Rag	—
27 Mar. '11	13	73.1	.80	1 9 1 10 1 12 3 13 1 14 2 15 1 16	13	Nil	Rat faeces crushed	3 opened and found to be empty ("false" cocoons)
9 June '11	20	76.0	.84	1 9 1 11 3 12 4 13 4 15 4 17 1 18 1 19 1 34	15	Nil	B.S. Rag	—
9 "	6	76.1	.87	3 11 1 16 1 22	14	Nil	Fla faeces	One cocoon opened empty ("false" cocoon)

* Experiments in Table XXXVI are for the most part in direct continuity with those dealing with the larval stage given in Table XXI, and up to 15 February 1911 with egg stage experiments given in Table V.

TABLE XXXVI.—Continued.

Incubator 85° Wet.									
Date of commence- ment of experiment in the (larval or cocoons found)		Temp.	Humidity	Approximate duration of cocoon stage		Average no. of days in cocoon	Mortality in cocoon stage	Food	Remarks
24 July '10	1	about 85.0	.72	1	23	—	Nil	B. S. Rag	—
6 Aug. '10	1	84.3	.75	1	9	—	Nil	B. S. Rag	—
26 Sept. '10	7	84.4	.72	1	6	7	43 ⁹ / ₁₀	Flea faeces	—
				2	7				
				1	11				
29 March '11	15	84.0	.74	1	6	12	11 ⁹ / ₁₀	Rat faeces crushed	Remainder (7 cocoons) opened, one found to contain dried up flea, remainder "false" cocoons
				1	8				
				3	12				
				2	14				
				1	21				
6 June '11	18	84.0	.81	1	7	13	Nil	B. S. Rag	2 cocoons opened and found empty ("false" cocoons)
				1	8				
				3	9				
				2	10				
				3	11				
				2	14				
				2	16				
				2	26				
7 "	9	84.2	.80	1	9	13	Nil	Flea faeces	1 cocoon opened and found empty ("false" cocoon)
				2	11				
				4	12				
				1	26				

Laboratory Cupboard.										
13 July '10	1	60	.84	1	22	—	Nil	Rat faeces	—	
1 Aug. '10	1	60	.84	1	43	—	Nil	Flea faeces	—	
11 "	6	Approx. only to 8 Nov. 60 max. min. Nov. 54.0 42.8 Dec. 56.5 47.8 Jan. 53.5 44.0 Feb. 52.8 43.6 Mar. 55.2 45.5 Apr. 58.2 48.2	.84 .84 .85 .84 .83 .85 .78	1 1 1 1 1 1	23 24 104 114 215 220	116	—	Nil	B.S. Rag	—
14 "	3	Approx. 60	.84	1	59	—	Nil	—	—	
20 "	10	Approx. to 8 Nov. 60 max. min. Nov. 54.0 42.8 Dec. 56.5 47.8 Jan. 53.5 44.0 Feb. 52.8 43.6 Mar. 55.2 45.5 Apr. 58.2 48.2	.84 .84 .85 .84 .83 .85 .78	1 1 1 1 1 5 opened	37 40 55 66 178	75	Nil	Flea faeces	22 July '11 5 opened and found to be empty (? false cocoons)	
1 Oct. '10	16	(as above)		3 1 4 1 1 1 5 opened	74 77 81 86 87 113	83	31 %	B.S. Rag	24 Aug. '11 5 opened and found to contain dried up larvae	
2 "	2	—	—	1	143	—	50 %	B.S. Rag	1 cocoon opened 21 Oct. '11 found to contain dried up pupa	

TABLE XXXVI.—Continued.

Date of commencement of experiment in the '1g (or larval) stage	No. of cocoons found	Temp. (as above to April)	Humidity	No. of cocoons	Approximate duration of cocoon stage	Average no. of days in cocoon	Mortality in cocoon stage	Food	Remarks
10 "	2	May 61.8 54.3 June 66.4 58.2	.78 .79	1	172	—	—	Bran	—
3 June '11	7	72.4 64.1	.73	3 2 2	13 19 20	17	Nil	B. S. Rag	—
12 "	13	71.1 63.1	.74	5 1 2 1 4	13 14 18 20 21	17	Nil	Flea faeces	—
14 Oct. '10	2	1911 max. min. Feb. 46.4 44.8 Mar. 46.5 45.3 Apr. 48.5 46.6 May 53.9 52.6	.92 .91 .91 .92	1 1	38 90	64	Nil	Bran	—
June '11	18	June 58.1 56.7 July 61.6 60.4 Aug. 64.5 63.4 Sept. 60.3 59.5	.93 .93 .94 .93	1 3 .3 8 1 2	13 18 19 20 26 opened	19	11%	B. S. Rag	2 opened 8 Nov. '11 contained 1 dried up larva, 1 dried up flea
12 "	17	—	—	4 1 2 2 1 4 1 1	6* 13 18 19 20 26 opened	27	6%	Flea faeces	1 cocoon opened 9 Nov. '11 contained living flea; 1 cocoon, opened 14 Dec. 1911, contained a dead flea

* Period may be 3 or 4 days longer owing to lapsus in observation.

TABLE XXXVII. *Coccons P. irritans*. "Continuous" series, contrasted experiments (compiled from Table XXXVI); larvae from same batch of eggs.

Date of commencement of experiment in egg stage	Place	No. of coccons found	Temp.	Humidity	Approximate duration of cocoon stage		Av. no. of days in cocoon	Mortality in cocoon stage	Food
					No. of coccons	Days			
11 Aug. '10	Incubator 75 Wet	4	75.0	.60	4	12	12	Nil	B.S. Rag
11 "	Laboratory Cupboard	6	Approximate only to 8 Nov.		1	23	116	Nil	B.S. Rag
			60	.84		24			
			max. min.						
			Nov. 54.0	42.8		1			
			Dec. 56.5	47.8		1			
			Jan. 53.5	44.0		1			
			Feb. 52.8	43.6		1			
			Mar. 55.2	45.5					
			Apr. 58.2	48.2					

TABLE XXXVIII. *Cocoon P. irritans. Larvae all reared and cocoons spun in Incubator 75 Wet; cocoons then removed to various situations.*

(a) Jan.—April 1911. Temperature of Incubator 75 Wet, 74.4, Humidity .77 for Jan., .70 for March and April.												
Date on which cocoons were placed under test conditions	No.	Place	Temp. max. min.	Humidity	Dates of emergence of fleas		No. of cocoons	Approximate duration of cocoon stage		Mortality	Remarks	
					1 on 21 March	2		1	77			—
3 Jan. '11	16	Beehive	Jan. 43.9 32.0 Feb. 43.8 30.7 Mar. 52.5 33.3	— — —	1	77	—	93 0/0			Remainder opened 17 Aug. '11 contained 1 dried up flea, 7 dried up pupae, 5 dried up larvae (2 "false" cocoons)	
2 March	2	Incubator 85 Wet	83.9	.71	3 11 1 12	2	7	8	20		1 cocoon opened 19 Sept. '11 contained dried up larva	
4 "	3				1 12	1	8					
12 "	4	" 85 Dry	84.0	.58	1 22 1 27	1	10	12	50		2 cocoons opened 19 Sept., 1 contained dried up flea, 1 contained dried up larva	
20 "	2	Lab. Cupboard	Mar. 52.2 45.5	.85	1 4 May	2	43	58	Nil		Remaining cocoons "false"	
19 April	5		Apr. 58.2 48.2	.78	1 27	1	45					
			May 61.8 54.3	.78	2 2 June	1	52					
			June 66.4 58.2	.79	1 10	1	68					
			July 71.5 62.7	.75	1 27 July	1	99					
27 March	3	Cellar	Mar. 46.5 45.3 Apr. 48.5 46.6 May 53.9 52.6 June 58.1 56.7 July 61.6 60.4 Aug. 64.5 63.4	.91 .91 .92 .93 .93 .94	1 26 June 1 16 Aug.	1	91 142	116	33		Remaining cocoon opened contained dead flea	
15 April	8	Incubator 75 Dry	75.2	.50	1 23 April 1 27 1 28 1 30	1	8 12 13 15	14	38		Remaining cocoons opened contained dried up larvae	
					1 9 May	1	24					
19 "	5	Warm Cupboard	Apr. 63.0 May 62.9 June 66.6	.57 .60 .65	1 29 1 30 1 2 June 1 7 1 9	1	40 41 43 48 50	44	Nil			

(b) Special experiment with cocoons taken from the same batch; larvae reared and cocoons spun in Incubator 75 Wet. (Temp. April—May 1911, 75° F., Humidity .82).

10 May '11	10	Incubator 75 Dry	75.0	.53	1 on 21 May 1 22 " 2 24 " 1 26 " 1 29 "	11 12 14 16 19	14	Nil	5 cocoons opened 16 May contained 4 living pupae and 1 larval skin
11—12 May	12	" 85 Dry	83.9	.60	3 21 " 5 22 " 1 24 " 2 26 "	9 10 12 14	10	8	1 cocoon opened 23 Oct. contained dried up larva
13—15 "	11	" 85 Wet	84.0	.76	5 22 " 3 25 " 2 26 " 6 1 June 2 2 " 4 6 " 1 30 " 1 2 Aug.	7 10 11 10 11 15 39 72	9	Nil	1 cocoon opened 2 Oct. empty ("false cocoon")
20—22 "	18	" 75 Wet	76.0	.86	8 10 June 2 18 " 1 20 " 1 27 " 1 5 July 1 27 " 1 9 Aug. 2 16 " 1 21 " 1 5 Sept. 1 16 Oct. 1 20 Nov. 1 22 Dec. 3 25 " 1 25 Jan.	22 30 32 39 47 69 82 89 94 109 150 170 202 205 236	18	Nil	Remaining cocoons opened 2 Oct. empty ("false" cocoons)
16—19 "	17	Lab. Cupboard	May 65.0 56.5 June 66.4 58.2	.78 .79			25	20	5 cocoons opened 21 Oct., 1 con- tained dried up larva, 2 contained dried up pupae, 2 empty "false" cocoons
16—19 "	16	Cellar	May 53.9 52.6 June 58.1 56.7 July 61.4 60.4 Aug. 64.5 63.4 Sept. 60.6 59.5	.92 .93 .93 .94 .93			139	Nil	Remaining cocoons opened 21 June '12, 2 "false" cocoons
20—22 "	12	Beehive	May 76.0 48.0 June 73.0 56.5 July 74.1 55.4	— — —	2 6 June 5 10 " 1 20 " 1 14 July 1 18 "	15 19 29 53 57	26	16	2 cocoons opened 25 Oct. contained 1 dried up flea, 1 dried up larva

TABLE XXXVIII.—Continued.

(c) Special experiment with cocoons taken from the same batch; larvae reared and cocoons spun in Incubator 75 Wet. (Temp. May—June 1911, 75° F., Humidity .84).

Date on which the cocoons were placed under test conditions	No. of cocoons	Place	Temp. max. min.	Humidity	Dates of emergence of fleas	No. of cocoons	Approximate duration of cocoon stage Days	Aver. Mortality	Remarks
23—26 May '11	52	Cellar			1 on 20 June	1	25	89 Apparently Nil	Remaining cocoons empty, i.e. false
			May 53.9 52.6	.92	3 26 "	3	31		
			June 58.1 56.7	.93	2 5 July	2	40		
			July 61.6 60.4	.93	3 12 "	3	47		
			Aug. 64.5 63.4	.94	5 27 "	5	62		
			Sept. 60.6 59.5	.93	4 1 Aug.	4	67		
			Oct. 56.3 54.8	.92	3 9 "	3	75		
			Nov. 50.7 48.9	.91	6 11 "	6	77		
			Dec. 48.7 47.4	.92	1 16 "	1	82		
					1 21 "	1	87		
					1 28 "	1	94		
					1 5 Sept.	1	102		
					2 1 Nov.	2	159		
					3 27 "	3	185		
					2 20 Dec.	2	208		
					1 29 "	1	217		
27—30 "	41	Lab. Cupboard	June 66.4 58.2	.79	8 17 June	8	18	53	15 Cocoons opened 2 July '12, 2 contained dried up fleas, 1 contained dried up pupa, 3 contained dried up larvae. 3 false cocoons
			July 71.5 62.7	.75	2 20 "	2	21		
			Aug. 72.0 64.1	.74	1 26 "	1	27		
			Sept. 65.7 55.7	.77	1 5 July	1	36		
			Oct. 59.0 50.9	.81	2 12 "	2	43		
					3 21 "	3	52		
					1 27 "	1	58		
					2 2 Aug.	2	64		
					2 9 "	2	71		
					5 16 "	5	78		
					2 30 "	2	92		
					1 6 Sept.	1	98		
					2 9 "	2	101		
31 May— 2 June	46	Beehive	June 73.0 56.5	—	1 6 June	1	4	44	32 6 larvae emerged from cocoons and died. Cocoons opened contained 2 dead fleas, 1 dead pupa, 6 dead larvae. Remaining cocoons empty (false)
			July 80.3 58.6	—	1 14 "	1	12		
			Aug. 80.2 57.6	—	3 20 "	3	18		
					1 24 "	1	22		
					1 14 July	1	42		
					2 18 "	2	46		
					4 24 "	4	52		
					6 29 "	6	57		
					2 2 Aug.	2	61		

8—9	"	25	Incubator 75 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TABLE XXXVIII.—Continued.

(d) Special experiment with cocoons taken from the same batch; larvae reared and cocoons spun in Incubator 75 Wet. (Temp. August 1911, 75.7° F., Humidity .83).

Date on which the cocoons were placed under test conditions	No. of cocoons	Place	Temp. max. min.	Humidity	Dates of emergence of fleas	No. of cocoons	Approximate duration of cocoon stage, Days	Aver.	Mortality	Remarks
1 Aug. '11	36	Cellar	Aug. 64.5 63.4 Sept. 60.3 59.5 Oct. 56.3 54.8 Nov. 50.7 48.9 Dec. 48.7 47.4 Jan. 47.2 45.9 Feb. 45.8 44.6 Mar. 49.1 48.1 Apr. 50.3 49.1	.94 .93 .92 .91 .92 .92 .92 .93 .93	4 on 21 Aug. 3 23 " 2 11 Sept. 1 16 Oct. 5 1 Nov. 1 9 " 1 20 " 1 7 Dec. 4 20 " 1 22 " 1 4 Jan. 2 25 " 1 31 " 1 6 Feb. 2 13 " 1 26 " 1 27 March	4 3 2 1 5 1 1 1 4 1 1 2 1 2 1 1	20 27 41 76 92 100 111 128 141 143 156 177 183 187 196 209 239	110	Nil	4 cocoons missing, probably they were slightly spun and deserted by the larvae and subsequently fell to pieces
5 "	39	Lab. Cupboard	Aug. 72.0 64.1 Sept. 65.7 55.7 Oct. 59.0 50.9 Nov. 54.9 46.1 Dec. 53.9 46.2 Jan. 52.5 44.9 Feb. 54.4 45.3	.74 .77 .84 .85 .87 .89 .86	8 16 Aug. 18 30 " 1 9 Sept. 1 13 Oct. 1 20 " 1 7 Dec. 1 22 " 1 4 Jan. 1 17 Feb.	8 18 1 1 1 1 1 1 1	11 25 34 69 76 124 139 152 196	40	10	Remaining cocoons opened 3 July '12 contained 1 dried up flea, 3 dried up larvae, 2 false cocoons
8 "	33	Beehive	Aug. 80.2 57.6 Sept. 75.3 48.0	— —	7 17 Aug. 12 22 " 1 26 " 1 28 " 1 9 Sept. 1 16 " 1 22 "	7 12 1 1 1 1 1	9 14 18 20 32 39 45	16	3	Remainder of cocoons opened 23 June '12 contained 1 dead larva, 8 false cocoons

TABLE XXXVIII.—Continued.

(c) Special experiment with cocoons taken from the same batch; larvae reared and cocoons spun in Incubator 75 Wet. (Temp. Nov.—Dec. 1911, 75° F., Humidity 70.)

Date on which the cocoons were placed under test conditions	No. of cocoons	Place	Temp.	Humidity	Dates of emergence of fleas	No. of cocoons	Approximate duration of cocoon stage	Aver.	Mortality	Remarks		
							Days					
6 Nov. '11	16	Incubator 93 Dry	92.9	.56	2 on 13 Nov. 2 16 " 1 1 Dec.	2 2 1	7 10 25	12	69	Cocoons opened 16 March '12 contained 7 dead fleas, remainder 4 dead larvae		
16 "	22	" 85/93 Wet	84.2 93.0	.72* .66	2 29 Nov.	2	13	—	Doubtful	Cocoons opened 11 May '12 contained 6 dead pupae, 4 dead larvae, 10 unaccounted for, presumably false cocoons		
17 "	16	" 75 Dry	74.5	.56	* Up to 29 Nov. '72, subsequently 2 5 Dec.	2	18	—	87	Cocoons opened 13 Dec. contained 14 dried up larvae		
21 "	19	" 75 Wet	Nov. 75.0 Dec. 75.8 Jan. 75.5 Feb. 75.0 Mar. 75.1 Apr. 75.8 May 75.6	.69 .65 .71 .76 .79 .79 .86	2 1 " 7 5 " 1 16 " 1 24 " 1 1 Jan. 1 16 May	2 7 1 1 1 1	10 14 25 33 41 146	28	21	Cocoons opened 4 July '12 contained 2 dried up fleas, 2 dried up larvae, 2 false cocoons		
21 "	19	Warm Cupboard	Nov. 63.0 Dec. 62.3 Jan. 59.7 Feb. 59.5	.58 .62 .60 .63	2 5 Dec. 1 7 " 2 11 " 2 13 " 1 18 " 1 22 " 1 26 " 1 4 Jan. 2 8 " 1 25 " 1 12 Feb.	2 1 2 2 1 1 1 1 2 1 1	14 16 20 22 27 31 35 44 48 65 83	34	10	Cocoons opened 4 July '12 contained 2 dried up larvae, 1 false cocoon		
23 "	17	Beehive	Nov. } 23 to } 42.0 32.7 30 }	—	No emergence					—	100	Cocoons opened 23 June '12; many of the cocoons very frail and in fragments, apparently there had been 5 false cocoons. There were 12 dead larvae

Dec. 46.8 35.0

Thermometer fell to 23° for three nights, Dec. 6—8

Thermometer fell to 23° for three nights, Dec. 6—8

28 "	24	Lab. Cupboard	Nov. 54-9	46-1	'85	1	29 Dec.	1	31	95	12	Cocoons opened 13 June '12 contained 1 dried up flea, 2 dried up larvae
			Dec. 53-9	46-2	'87	1	4 Jan.	1	37			
			Jan. 52-5	44-9	'89	1	8 "	1	41			
			Feb. 54-4	45-3	'86	3	25 "	3	58			
			Mar. 56-0	47-8	'76	2	17 Feb.	2	81			
			Apr. 60-6	50-4	'83	1	20 "	1	84			
						1	29 "	1	93			
						1	5 March	1	98			
						1	11 "	1	103			
						1	25 "	1	118			
						3	28 "	3	121			
						2	1 April	2	125			
						1	20 "	1	144			
						1	24 "	1	148			
						1	30 "	1	154			
7 Dec.	31	Cellar	Dec. 48-7	47-4	'92	1	31 Jan.	1	55	115	32	Cocoons opened 22 June '12 contained 10 dead fleas, 3 false cocoons
			Jan. 47-2	45-9	'92	2	6 Feb.	2	61			
			Feb. 45-8	44-6	'92	2	13 "	2	68			
			Mar. 49-1	48-1	'93	2	7 March	2	91			
			Apr. 50-3	49-1	'93	1	13 "	1	99			
			May 54-2	53-2	'93	1	16 April	1	131			
			June 56-4	55-6	'93	1	26 "	1	141			
						1	3 May	1	148			
						1	9 "	1	154			
						2	16 "	2	161			
						1	21 "	1	166			
						2	25 "	2	170			
						1	4 June	1	180			

(f) Special experiment with cocoons taken from the same batch; larvae reared and cocoons spun in Incubator 75 Wet. Feb. — March, 1912. (Temperature 75° F., Humidity 76.)

Date	Time	Incubator	Wet	°C	5	5 March	5	8	10	33	Cocoons opened 2 July contained 6 dried up fleas, 1 dried up larva, 2 false cocoons. 1 unaccounted for
26 Feb. '12				92.6	5	5 March	4	12			
					4	9 "	4	15			
					2	12 "	2				
28 "	11	"	75 Wet	75.6	10	12 "	10	13	13	Nil	
					1	14 "	1	15			
1 Mar. '12	10	"	75 Dry	73.2	2	14 "	2	13	16	20	Cocoons opened 2 July contained 2 dried up larvae, 1 false cocoon
					4	18 "	4	17			
					1	22 "	1	21			

TABLE XXXVIII.—Continued.

(g) Emergence of adults from stock of cocoons kept in Laboratory Cupboard, larvae reared and cocoons spun in Laboratory Cupboard.

Dates when cocoons were found	Temperature max. min.	Humidity	Dates of emergence	Average 143 days	Approx. no. of days in cocoon
1 on Sept. '10	Sept. '10 about 60	.84	1 on 27 Jan. '11		1 in 134
3 21 "	Oct.	.84	4 27 Feb. '11		1 155
2 25 "	Nov.	.84	2 1 March '11		* 1 157
Remainder during October	Dec.	.85	1 2 "		3 159
	Jan. '11	.84	2 3 "		1 121
	Feb.	.83	2 4 "		1 122
	March	.85	1 9 "		1 123
	April	.78	2 20 "		2 124
			1 23 "		+ 1 129
			1 1 April '11		2 140
			1 18 "		1 143
			1 21 "		1 151
					1 169
					1 172

* These were assumed to have emerged from the Sept. cocoons.

+ All dates calculated from 31 Oct. '11. One cocoon was kept apart so that its date of emergence can be definitely fixed at 169 days.

allowed to spin under one set of conditions and transferred to contrasting conditions for the cocoon stage.

Date on which larvae were removed from incubator	No. of larvae	Place in which cocoons formed and date when found and transferred	Temperature max. min.	Humidity	Place to which cocoons were transferred and date of emergence of fleas	Temperature 84-7	Humidity -80	Approximate duration of cocoon stage		Mortality	Remarks
								No. of cocoons	Days		
27 April '11	10	Beehive 1 18 May 1 26 " Remainder of larvae dead	69-1 43-7	—	Incubator 85 Wet 1 29 May 1 6 June	84-7	-80	2	11	—	Nil
27 April	10	Incubator 85 Wet 2 2 May 3 4 " 3 5 " Remainder of larvae dead	83-5	.75	Beehive 2 30 May 2 16 June 1 20 "	max. min. May 71-8 45-2 June 73-0 56-5	—	2	28	23	37% 3 cocoons opened 30 Sept. contained 1 dried up flea, 2 dried up larvae
2 May	10	Lab. Cupboard 1 9 May 2 13 " 1 23 " 1 27 " 1 1 June 1 larva emerged from cocoon and failed to spin again Remainder of larvae dead	61-8 54-3	.78	Incubator 75 Dry 2 23 May 1 7 June 1 13 "	75-3	.53	1	10	17	20% Remaining cocoons opened 23 Oct. contained dried up larvae
27 April	10	Cellar 1 9 May 2 18 " 2 27 " 1 3 June 1 8 " 2 larvae emerged from cocoons and failed to spin again Remainder of larvae dead	54-8 53-4	.92	Incubator 85 Dry 1 20 June 1 2 Aug.	84-1	.61	1	41	58	60% Remaining cocoons opened 2 Oct. contained dried up larvae
2 May	10	Incubator 85 Dry 7 8 May 1 11 " Remainder dead	84-3	.59	Cellar 2 3 June 2 20 " 1 12 July 1 1 Aug.	May 53-9 52-6 June 58-1 56-7 July 61-6 60-4	.92 .93 .93	2	26	48	Nil Remaining cocoons opened and found empty (false cocoons)
2 May	16	Incubator 75 Wet 4 5 May 9 8 " 3 10 " 1 12 "	74-4	.83	Warm Cupboard 1 19 May 1 21 " 1 23 " 1 24 " 1 26 " 2 29 " 4 31 " 1 1 June 2 6 " 1 8 "	May 62-9 June 66-6	.60 .65	1	14	22	12% 1 larva came out of cocoon and died, 1 cocoon opened 30 Sept. contained dried up larva

TABLE XL. *Cocoon stage Ct. canis.*

(a) Larvae taken from dog's bed placed in large glass tubes and allowed to spin and to pass the cocoon stage in the same place (1) in Incubator 75° Wet and (2) in the Cellar.

Date on which larvae were put into tubes	No. of cocoons	Place to which cocoons were transferred	Temperature	Humidity	Date of emergence of fleas	Mortality	Remarks
26 April '11	17 cocoons found	Incubator 75° Wet	April 75.0 May 74.9 June 75.8	.78 .85 .86	1 on 8 May '11 3 10 " 1 23 " 1 27 " 3 1 June '11 14 6 " 1 8 "	*	—
"	28 cocoons found	Cellar	April 48.5 May 53.9 June 58.1 July 61.6 Aug. 64.5	46.6 52.6 56.7 60.4 63.4	.91 .92 .93 .93 .94	2 5 July '11 7 12 " 4 3 Aug. '11 1 9 " —	—

(b) Larvae taken from dog's bed and allowed to spin in Laboratory Cupboard, November 1910; cocoons then removed to various situations.							
Date on which cocoons were placed under test conditions	No. of cocoons	Place to which cocoons were transferred	Temperature	Humidity	Date of emergence of fleas	Mortality	Remarks
17 Nov. '10†	7	Incubator 85° Wet	83.9	.71	1 23 Nov. '10 5 25 "	Nil	1 cocoon opened and found to be empty ("false" cocoon)
"	7	" 75° Wet	74.2	.73	1 25 " 3 30 "	14 %	2 cocoons opened found to be empty (1 dead flea, 1 "false" cocoon)
"	6	Warm Cupboard	max. 67.5 min. 61.0	.54	2 4 Dec. '10 1 7 " 2 8 "	16 %	1 cocoon opened found to contain dead flea
"	7	Cellar	48.6	.92	1 13 Feb. '10 1 18 "	71 %	5 cocoons opened contained dead fleas

* NOTE. No accurate figures of mortality can be given.

† There is some doubt as to the correct date of spinning, the date given is the date of transfer only, the number of days in cocoon is not definite.

(c) Larvæ taken from dog's bed and allowed to spin in Incubator 75° Wet (Temperature 76° F., Humidity 84); cocoons then removed to various situations.

Date on which cocoons were placed under test conditions	No. of cocoons	Places to which cocoons were transferred	Temperature	Humidity	Date of emergence of fleas		Approximate duration of cocoon stage	Average	Remarks
					No. on 15 Aug. '11	No. of cocoons			
31 July '11	about 100	Incubator 75° Wet	74·9	·77	85 on 15 Aug. '11	85	15	16	Remaining cocoons opened 16 Dec. '11 contained dried up larvæ
"	"	"	75·7	·59	7 17 "	7	17		
					1 18 " " '11	1	18		
					2 12 Oct. '11	2	72		
					78 14 Aug. '11	78	14		
					1 25 "	1	25		
					1 28 "	1	28		
					1 4 Sept. '11	1	35		
"	100	"	81·8	·79	77 14 Aug. '11	77	14	15	Remaining cocoons opened 16 Dec. '11 contained dried up fleas
					1 21 "	1	21		
					1 28 "	1	28		
					1 12 Sept. '11	1	43		
					1 19 "	1	50		
"	100	"	84·0	·61	80 14 Aug. '11	80	14	15	Remaining cocoons opened 16 Dec. '11 contained dried up fleas
					3 25 "	3	25		
					1 19 Sept. '11	1	50		
"	100	Cellar	max. 64·5 min. 63·4	·94	12 16 Aug. '11	12	16	130	Remaining cocoons opened 7 Sept. '12 contained 2 dead fleas
			Aug. 60·6 59·5	·93	21 28 " " '11	21	28		
			Sept. 56·3 54·8	·92	9 11 Sept. '11	9	42		
			Nov. 50·7 48·9	·91	3 19 " " '11	3	50		
			Dec. 48·7 47·4	·92	1 16 Oct. '11	1	80		
			1912		2 9 Nov. '11	2	101		
			Jan. 47·2 45·9	·92	6 20 Dec. '11	6	142		
			Feb. 45·8 44·6	·92	3 22 "	3	144		
			Mar. 49·1 48·1	·93	2 25 "	2	147		
			April 50·3 49·1	·93	1 29 " " '12	1	151		
			May 54·2 53·2	·93	4 4 Jan. '12	4	157		
			June 56·4 55·6	·93	11 10 " " '12	11	163		
			July 60·1 59·3	·93	8 6 Feb. '12	8	190		
					1 19 "	1	203		
					3 26 " " '12	3	210		
					6 21 Mar. '12	6	234		
					5 27 "	5	240		
					3 3 April '12	3	248		
					2 10 "	2	258		
					4 26 " " '12	4	270		
					2 9 May '12	2	283		
					2 25 "	2	299		
					1 19 July '12	1	354		

TABLE XL.—*Continued.*

Date on which cocoons were placed under test-conditions 31 July '11	No. of cocoons about 100	Place to which cocoons were transferred * Lab. Cupboard	Temperature		Humidity	Date of emergence of fleas		No. of cocoons	Approximate duration of cocoon stage Days	Average	Remarks
			max.	min.		80 on 16 Aug. '11	5 30 "				
			Aug. 72.0	64.1	.74	5 30 "	5 30 "	80	16	67	Cocoons opened, no dead found
			Sept. 65.7	55.7	.77	2 2	6 Sept. '11	2	5	30	
			Oct. 59.0	50.9	.84	1 19 Dec. '11	1 19 Dec. '11	1	141	37	
			Nov. 54.9	46.1	.85	1 22 "	1 22 "	1	141	141	
			Dec. 53.9	46.2	.87	1 25 "	1 25 "	1	147	144	
			Jan. 52.5	44.9	.89	2 10 Feb. '12	2 10 Feb. '12	2	194	147	
			Feb. 54.4	45.3	.86	1 17 "	1 17 "	1	201	201	
			Mar. 56.0	47.8	.76	3 20 "	3 20 "	3	204	204	
			April 60.6	50.4	.83	1 23 "	1 23 "	1	207	207	
						3 5 Mar. '12	3 5 Mar. '12	3	218	218	
						3 11 "	3 11 "	3	224	224	
						1 21 "	1 21 "	1	234	234	
						1 25 "	1 25 "	1	238	238	
						1 28 "	1 28 "	1	241	241	
						2 1 April '12	2 1 April '12	2	245	245	
						3 14 "	3 14 "	3	258	258	
						3 24 "	3 24 "	3	268	268	
						1 30 "	1 30 "	1	274	274	
"	100	Beehive	80.2	57.6	—	20 14 Aug. '11	20 14 Aug. '11	20	14	18	Cocoons opened contained 7 dried up fleas
						48 18 "	48 18 "	48	18	22	
						26 22 "	26 22 "	26	22	26	
						1 26 "	1 26 "	1	26	26	
"	100	Warm Cupboard	Dry			80 14 "	80 14 "	80	14	16	Remaining cocoons opened 13 Dec. '11 contained dried up fleas
			Aug. 75.0		.66	5 17 "	5 17 "	5	17	17	
			Sept. 66.3		.66	7 22 "	7 22 "	7	22	22	
			Oct. 67.4		.59	1 23 Sept. '11	1 23 Sept. '11	1	54	54	
						1 25 "	1 25 "	1	56	56	
						1 12 Oct. '11	1 12 Oct. '11	1	73	73	

Note. No accurate figures of mortality can be given.

(d) Larvae taken from a dog's bed and allowed to spin in Incubator 75 Wet; cocoons then removed to various situations.

Date on which cocoons were placed under test conditions	No. of cocoons	Place to which cocoons were transferred	Temperature	Humidity	Date of emergence of fleas	Number of days in cocoon	Average	Remarks
8 July '12	50 approx.	Incubator 93 Wet	94.8	.82	1 on 13 July '12 3 19 "	5 11	9	Cocoons opened 4 Sept. contained 4 dead fleas, 22 dead pupae, 11 dead larvae
12 "	50 "	" 93 Dry	93.0	.57	21 19 " 6 24 " 2 28 "	7 12 16	8	Cocoons opened 4 Sept. contained 10 dead fleas, 2 dead pupae, 6 dead larvae
12 "	50 "	" 75 Wet	75.0	.86	4 18 " 38 24 " 2 28 " 1 4 Aug. '12	6 12 16 23	12	Cocoons opened 5 Sept. all empty, probably no mortality
12 "	50 "	" 75 Dry	76.2	.64	2 19 July '12 39 24 " 2 28 " 1 1 Aug. '12	7 12 16 20	12	Cocoons opened 5 Sept. contained 1 dead larva, remainder empty
12 "	50 "	* Cellar	max. min. July 60.1 59.3 Aug. 57.5 56.5 Sept. 56.0 54.3	.93 .93 .92	+10 7 Sept. '12 1 28 " +2 11 Oct. '12 3 14 " +12 14 " 1 17 " 1 31 " 1 13 Nov. '12 1 21 " 1 23 " 1 19 Dec. '12 1 21 " 1 17 Feb. '13 1 25 Mar. '13 7 emerged between May and July	57 78 91 94 94 97 111 124 132 134 160 162 220 256 say 292	129	1 dead flea found in cocoon, remaining cocoons empty

* No record of temperature or humidity kept subsequently to Sept. 1912.

+ Taken out of cocoons.

NOTE. No accurate figures of mortality can be given.

TABLE XL.—Continued.

Date on which cocoons were placed under test conditions	No. of cocoons	Place to which cocoons were transferred	Temperature max. min.	Humidity	Date of emergence of fleas	Number of days in cocoon	Average	Remarks
12 July '12	50	* Lab. Cupboard	July 68.5 62.1 Aug. 63.3 56.3 Sept. 61.5 53.0	July -79 Aug. -84 Sept. -86	2 27 July '12 8 2 Aug. '12 4 9 " 2 30 " +2 6 Sept. '12 1 30 " 1 20 Nov. '12 3 21 Dec. '12 4 4 Feb. '13 1 9 " 1 5 Mar. '13 3 8 " 1 25 " 10 emerged subsequently	15 At least 133 21 28 49 56 65 116 147 192 197 221 224 241 say 250		Remaining cocoons empty
15 "	50	Warm Cupboard	July 67.3 Aug. 64.5	July -71 Aug. -71	1 26 July '12 5 30 " 6 3 Aug. '12 13 7 " 4 9 " 2 12 " 1 25 " 3 30 " 9 5 Sept. '12 2 9 Aug. '12 2 28 " 1 3 Sept. '12 2 6 " 6 17 Oct. '12 1 30 " 2 8 Nov. '12 2 4 Feb. '13 1 9 " 3 5 Mar. '13 3 emerged subsequently	11 15 19 23 25 28 41 46 52 25 44 50 53 94 107 116 204 209 233 say 240	29	Cocoons opened 5 Sept. contained 9 live fleas, 5 dead fleas, 1 dead larva; 2 dead fleas from 1 cocoon. Several cocoons spun back to back, in one of these double cocoons one compartment contained a dead flea and the other a live one
15 "	50	* Beehive	July 73.4 55.4 Aug. 64.9 45.6 Sept. 63.2 45.7	— —	2 9 Aug. '12 2 28 " 1 3 Sept. '12 2 6 " 6 17 Oct. '12 1 30 " 2 8 Nov. '12 2 4 Feb. '13 1 9 " 3 5 Mar. '13 3 emerged subsequently	129		Some cocoons opened 6 Sept. contained 2 living fleas Remaining cocoons opened 13 July '13 contained 1 dead larva, remainder empty

* No record of temperature or humidity kept subsequently to Sept. 1912.

† Taken out of cocoons 6 Sept. '12.

NOTE. No accurate figures of mortality can be given.

TABLE XLI. *Cocoons C. gallinae. Eggs laid by adults fed on human blood; larvae reared and cocoons spun in Incubator 75 Wet; cocoons then removed to various situations.*

Date on which cocoons were placed under test conditions	No. of cocoons	Place to which cocoons were transferred	Temperature	Humidity	Date of emergence of fleas	No. of days in cocoon	Average	Mortality	Remarks
25 June '12	14	Incubator 75 Dry	June 75.8 July 76.2 Aug. 75.0	.54 .64 .56	8 8 July 3 13 " 1 24 Aug.	13 18 60	18	7 %	6 larvae emerged from cocoons, 5 pupated and fleas emerged, 1 failed to pupate Cocoons opened 5 Sept. and found to be empty, "false" cocoon
"	14	Incubator 93 Wet	95.9	.70	1 2 July	7	—	85 %	1 larva emerged from its cocoon but successfully pupated and emerged Remaining cocoons opened 4 Sept., 11 dead pupae, 1 dead larva, 1 empty "false" cocoon
28 June	14	Incubator 93 Dry	93.0	.55	2 8 July	10	—	(Total) 93 % (In cocoon) 5 %	5 larvae emerged from their cocoons but pupated after a few days. Pupae subsequently dried up Remaining cocoons opened 4 Sept., 7 dead pupae, 1 dead larva
"	11	Cellar	max. min. June 56.5 55.6 July 60.1 59.3 Aug. 57.5 56.5	.93 .93 .93	4 26 July 1 3 Aug. 4 9 " 2 6 Sept.	28 36 42 70	41	Nil	2 larvae emerged from cocoons but pupated Remaining cocoons opened 6 Sept., 2 living fleas found
2 July	11	Beehive	July 73.4 55.4 Aug. 64.9 45.6 Sept. 63.2 45.7	— — —	1 15 July 4 26 " 1 2 Aug. 1 9 " 1 24 " 1 3 Sept. 1 6 "	13 24 31 38 53 63 66	36	(Total) 9 % (In cocoon) Nil	1 larva emerged from its cocoon and died

TABLE XLII. *Cocoon* *L. musculi*. Larvae taken from breeding cages, cocoons spun in Incubator 75° Wet (Temperature 75° F., Humidity .85), and afterwards removed to various situations.

Date on which cocoons were placed under test conditions	No. of cocoons	Place to which cocoons were transferred	Temperature	Humidity	Date of emergence of fleas	No. of days in cocoon	Average	Mortality	Remarks
18 May '12	24	Incubator 75° Wet	May 75.6 June 74.9 July 75.0 Aug. 74.3	.86 .83 .86 .81	1 28 May 11 31 " 1 3 June 1 24 Aug. 1 30 "	10 13 16 98 104	24	37 %	Remainder of cocoons opened 5 Sept., 1 dead flea, 6 dead pupae, 2 dead larvae
"	24	Incubator 93° Wet	May 95.1 June 94.5 July 95.2 Aug. 94.2	.75 .77 .74 .75	None emerged	—	—	87 % (at least)	Cocoons opened 4 Sept., 20 dead larvae, 1 dead pupa, 2 resting larvae (109 days) Resting larvae and remaining cocoon transferred to Incubator 75° Wet, both larvae died, cocoon a "false", one
"	24	Cellar	max. May 54.2 53.2 June 56.4 55.6 July 60.1 59.3	.93 .93 .93	7 18 June 8 27 " 3 5 July 1 15 " 20 24 " 4 28 " 1 19 " 12 24 " 6 27 " 3 30 " 2 7 Aug.	31 40 48 58 12 16 7 12 15 18 26	38	Nil	2 cocoons opened 4 Sept., 2 living pupae, which subsequently died (109 days) Remaining cocoons opened and found empty, "false" cocoons
12 July	25	Incubator 75° Wet	75.0	.88	20 24 " 4 28 " 1 19 " 12 24 " 6 27 " 3 30 " 2 7 Aug.	12 16 7 12 15 18 26	13	Nil	Remaining cocoon opened 5 Sept. contained living pupa (55 days)
"	25	Incubator 75° Dry	July 76.2 Aug. 75.0	.64 .56	1 19 " 12 24 " 6 27 " 3 30 " 2 7 Aug.	7 12 15 18 26	14	Nil	Remaining cocoon opened 5 Sept. empty, "false" cocoons
"	25	Incubator 93° Dry	July 93.0 Aug. 93.0	.57 .54	None emerged	—	—	60 % (at least)	Cocoons opened 4 Sept., 12 dead larvae, 2 dead pupae, 2 resting larvae, 1 shrunken larva but still fresh and moist, 1 empty 7 cocoons and 2 resting larvae transferred to Incubator 75° Wet, the larvae died and the cocoons proved to be empty (? "false")

"	25	Incubator 93 Wet	95.2	.74	None emerged	—	—	100 %	Cocoons opened 10 Sept., 14 dried up larvae, 6 dried up pupae, 5 dried up fleas
"	25	Cellar	max. min. July 60.1 59.3 Aug. 57.5 56.5 Sept. 56.0 54.3	.93 .93 .92	5 1 Aug. 11 9 " 3 13 " 1 21 " 1 6 Sept.	20 28 32 43 56	29	Nil	Remaining cocoons opened 6 Sept., 1 living flea, 1 living pupa, 3 empty, "false" cocoons (56 days)
"	25	Lab. Cupboard	July 68.5 62.1 Aug. 63.3 56.3	.79 .84	5 27 July 8 30 " 8 2 Aug. 3 9 "	15 18 21 28	19	Nil	Remaining cocoons opened 6 Sept., 1 resting larva (56 days)
"	25	Warm Cupboard	July 67.3 Aug. 64.5	.71 .71	7 26 July 5 30 " 5 3 Aug. 1 7 " 1 19 " 1 28 " 1 30 " 1 28 Sept.	14 18 22 26 38 47 49 78	24	Nil	Two cocoons opened 5 Sept., 10 empty, 1 living pupa, 1 resting larva (55 days), subsequently pupated and flea emerged 28 Sept., remaining cocoons "false"
"	25	Beehive	July 73.4 55.4 Aug. 64.9 45.6	— —	4 26 July 13 2 Aug. 4 9 " 1 12 "	14 21 28 31	21	Nil	Cocoons opened 6 Sept., 1 resting larva (56 days), 2 empty, "false" cocoons

TABLE XLIII. *Retarded emergence from cocoons C. fasciatus; full grown larvae taken from the breeding cages and placed with food to complete their development in Incubators 75 Wet and 85 Wet respectively.*

[illegible]

ii. *Influence of immersion in water upon the vitality of Cocoons. Ct. canis* (Table XLIV).

An attempt was made to ascertain what powers, if any, fleas might possess of surviving inundation. The cocoon stage seems to afford the only chance of fleas being able to survive actual flooding of their habitat. A plentiful supply of the cocoons of *Ct. canis* induced the selection of this species for a trial. The larvae were allowed to spin at the bottom of card jars and, after allowing a few days for the silk to harden, the loose sand was emptied out and water poured in until the cocoons were thoroughly submerged.

The water was poured off from the various jars after periods of half an hour, three quarters of an hour, one hour, 12 hours and one week; they were then placed to dry and the fleas allowed to emerge in the laboratory cupboard. As the trial took place during August and September 1911, emergence would be in response to natural conditions of heat and moisture apart from the wetting.

It will be seen that the cocoons of *Ct. canis* easily withstood 12 hours' soaking but were destroyed by a week's submergence. Too much reliance must not be placed on the difference in the number of emergencies in the different experiments as the numbers put in were not accurately counted, but only roughly estimated by dividing a large batch of larvae.

TABLE XLIV. *Immersion Test. Cocoons Ct. canis.*

Date 1911	Approximate number	Period under water	Emergence
1 Aug.	100	half-an-hour	98 fleas on 17 Aug. 2 " 30 " 5 " 28 Sept.
1 "	100	three-quarters of an hour	80 " 17 Aug. 12 " 30 " 1 " 28 Sept.
1 "	100	one hour	66 " 15 Aug. 15 " 30 " 6 " 28 Sept.
1 "	100	12 hours	62 " 17 Aug. 1 " 30 " 3 " 28 Sept.
10 "	100	one week	No emergence; two opened contained 1 dried up flea, 1 dried up pupa

iii. *Influence of external conditions at different periods of larval existence upon the duration of the cocoon stage C. fasciatus.*

Experiments were undertaken with a view to discover if the length of the resting period of the larvae of *C. fasciatus* within the cocoon was influenced by changes in the conditions of heat and humidity during the active period of larval life, and also to ascertain if possible at what period of growth the larvae could be most readily influenced.

Two batches of larvae were sorted out from a cage, one judged to be about half grown and the other ready to spin their cocoons. Both lots were subjected to similar treatment but otherwise kept apart, they are referred to as "full grown" and "half grown" respectively. These batches were subdivided, one half of each batch being allowed to feed and spin in incubator 75 Wet while the other half completed its development to the cocoon stage in receptacles kept in the cage from which they had been taken.

In both series of experiments the cocoons as formed were further separated into two batches, one batch of each lot being transferred to incubator 93 Dry and the other being allowed to remain where the larvae had spun. There are therefore eight records to follow up, viz. four batches in incubator 93 Dry, consisting of cocoons from "half grown" larvae spun in the cage and incubator 75 Wet and from "full grown" spun in the cage and incubator 75 Wet: two batches, one from "half grown" and one from "full grown" larvae maintained in incubator 75 Wet throughout and a similar pair kept in the cage.

The drastic conditions prevailing in incubator 93 Dry produced such a high mortality that the experiment more or less failed of its original purpose and a second series of trials were started on the same lines with the exception that incubator 93 Wet was substituted for 93 Dry.

A conclusion which may apparently be gleaned from the results of the first series is that the transference of "half grown" larvae from the cages to incubator 75 Wet is an unfavourable preparation for a higher temperature and drier atmosphere in the cocoon stage (compare batches B and D). I think further it may be taken as settled that a rise in temperature during active larval life, such as removal from the cage to incubator 75 Wet, does not act as a stimulus to lengthen the cocoon period. In fact it seems that the moderate conditions in incubator 75 Wet induce the individuals to curtail their resting period and to develop and emerge after a comparatively

short rest. Hot dry conditions appear to inhibit the development of individuals predisposed to a long rest but are not necessarily fatal, provided a reduction in temperature occurs within three or four months. The cage conditions on the other hand allow full scope for any natural predisposition of the species for a long cocoon period.

It is possible that extremes of climate may only control the prevalence of the adults of this species by rendering a distributed emergence impossible and inducing a condensed periodic one in its place.

The results of the second series, while fully in accord with the above conclusions, tend to support the phenomena brought to light in the monthly cocoon tests with this species, Table XXXIII; that this species has a fluctuating, or periodic, constitution as regards its powers of endurance. Allowance must however be made for the difference in incubator conditions in winter and summer brought to light in the newly hatched larvae trials (see footnote on p. 459 in reference to humidity conditions in incubators).

FIRST SERIES. "*Half grown*" larvae of *C. fasciatus* taken from cage and allowed to finish feeding and spin their cocoons in Incubator 75 Wet (temperature about 75° F., humidity about 70).

NOTE. The numbers giving the days spent in the cocoon do not increase in exact sequence in all cases owing to the fact that the cocoons were transferred in batches and the fleas as they emerged were presumed to have come from the earliest available batch.

Batch A left in Incubator 75 Wet. Temperature about 75° F.
Humidity about 65 to 70.

Date	Number of cocoons	Emergence of fleas	Approximate number of days in cocoon	Number of days in cocoon calculated from average date given in col. 1
22—29 Nov. '11	27	2 on 28 Nov.	6	4
(average date		2 1 Dec.	9	7
24 Nov.)		8 5 „	13	11
		9 11 „	{ 6 16	17
			{ 3 19	
		2 12 „	17	18
		1 11 Jan.	47	48
		1 23 „	55	60

2 cocoons opened 4 July '12 found empty ("false" cocoons).

Batch B transferred to Incubator 93 Dry. Temperature about 93° F.
Humidity about 56 to 60.

Date	Number of cocoons	Emergence of fleas	Number of days in cocoon
22—29 Nov. '11	27	(None)	—

On the 16 March '12, 19 cocoons were opened and found to contain 13 dead fleas, 5 dead larvae, 1 resting larva.

The remaining cocoons and 1 resting larva were transferred to Incubator 75 Wet; the larva died.

On the 8 July '12, all these cocoons were opened and found to contain dried up larvae.

FIRST SERIES. "*Half grown*" larvae of *C. fasciatus* taken from cage and allowed to finish feeding and to spin cocoons in the cage.

Batch C left in Cage.

Date	Number of cocoons	Emergence of fleas	Approximate number of days in cocoon	Number of days in cocoon calculated from average date given in col. 1
13 Dec. '11—	30	1 on 9 Jan. '12	say 27	16
19 Jan. '12		3 8 Feb.	„ 57	46
(average date		1 26 March	„ 103	92
24 Dec.)		1 1 April	„ 109	98
		1 23 „	„ 131	120
		1 27 „	„ 135	124
		3 20 June	„ 189	178
		4 1 July	„ 191	189
		1 3 „	„ 190	191
		1 10 Aug.	„ 221	229
		1 13 „	„ 224	232
		3 9 Sept.	„ 251	259
		1 15 „	„ 257	265

Remaining cocoons opened 25 July '13, all were empty except two which contained living larvae that had been resting for a period of from 563 to 590 days.

Batch D transferred to Incubator 93 Dry. Temperature about 93° F.

Humidity about .56 to .60.

Date	Number of cocoons	Emergence of fleas	Number of days in cocoon
13 Dec. '11—9 Jan. '12	30	1 on 20 Dec. '11	say 7

On 16 March '12, after a period of about 76 days, 6 cocoons were opened and found to contain 1 dead flea, 1 dead larva, 4 resting larvae.

The remaining cocoons and resting larvae were transferred to Incubator 75 Wet and there emerged

			Total number of days in cocoon including those spent in incubator 93 Dry
2 fleas on 3 April	18 days at 75° F., Humidity .79		94
1 flea 6 „	21 „ „		97
2 fleas 8 „	23 „ „		99
1 flea 11 „	26 „ „		102
1 „ 19 „	34 „ „		114

Remaining cocoons opened 3 July '12 contained 5 dried up fleas, 1 dried up larva, 14 empty cocoons ("false" cocoons!).

¹ The large number of "false" cocoons in these high temperature trials is accounted for by the larvae emerging and dying outside the cocoons where they become brittle and break into unrecognisable fragments.

FIRST SERIES. "*Full grown*" larvae of *C. fasciatus* taken from cage and allowed to finish feeding and to spin their cocoons in Incubator 75 Wet (temperature about 75° F., humidity about .70).

Batch A left in Incubator 75 Wet. Temperature about 75° F. Humidity about .65 to .70.

Date	Number of cocoons	Emergence of fleas	Number of days in cocoon
18 Nov. '11	31	6 on 28 Nov. '11	10
		4 1 Dec. '11	13
		1 5 "	17
		2 16 "	28
		2 1 Jan. '12	44
		2 4 "	47
		1 6 "	49
		2 8 "	51
		4 9 "	52
		1 11 "	54
		1 12 "	55
		2 15 "	58
		1 23 "	66
		2 26 "	69

Batch B transferred to Incubator 93 Dry. Temperature about 93° F.
Humidity about .56 to .60.

Date	Number of cocoons	Emergence of fleas	Number of days in cocoon
18 Nov. '11	31	3 on 25 Nov. '11	7
		1 1 Dec. '11	13

On the 16 March '12, after 119 days in Incubator 93 Dry, 6 cocoons were opened and found to contain 3 dead fleas, 3 resting larvae.

The resting larvae and balance of the cocoons were transferred to Incubator 75 Wet and there emerged

			Total no. of days in cocoon including 119 spent in Incubator 93 Dry
3 fleas on 1 April '12	3	16 days at 75° F., Humidity about .80	135
6 " 3 "	6	18 " " "	137
4 " 6 "	4	21 " " "	140
1 flea on 8 "	1	23 " " "	142

Cocoons opened 3 July '12, 2 dried up fleas, 2 dried up pupae, 4 dried up larvae.

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FIRST SERIES. "*Full grown*" larvae of *C. fasciatus* taken from cage and allowed to finish feeding and to spin in the breeding cage.

Batch C left in Cage.

Date	No. of cocoons	Emergence of fleas	Approximate no. of days in cocoon	No. of days in cocoon calculated from average date given in col. 1
19 Nov.—	32	1 on 28 Nov. '11	say 9	3
1 Dec. '11		1 20 Dec. '11	" 31	25
(average date		1 13 Jan. '12	" 55	49
25 Nov.)		2 23 April '12	" 144	150
		1 27 "	" 148	154
		1 24 May '12	" 175	184
		2 20 June '12	" 202	208
		1 1 July '12	" 213	219
		1 3 "	" 215	221
		3 26 "	" 238	244
		1 8 Aug. '12	" 251	257
		3 9 Sept. '12	" 283	289
		4 15 "	" 289	299
		1 19 Jan. '13	" 415	421

3 fleas, date of emergence unrecorded, found dead 25 July '13

Remaining cocoons opened 25 July '13 contained 1 dead and 3 living larvae; these latter had "rested" from 571 to 613 days.

Batch D transferred to Incubator 93 Dry. Temperature about 93° F.

Humidity about .56 to .60.

Date	Number of cocoons	Emergence of fleas	Number of days in cocoons
19 Nov.—1 Dec. '11	32	None	—

On 16 March '12, after about 112 days in Incubator 93 Dry, 3 cocoons were opened and found to contain resting larvae.

The resting larvae and remaining cocoons were transferred to Incubator 75 Wet and there emerged

				Total no. of days in cocoon including those (112 approximately) spent in Incubator 93 Dry
3 fleas on 1 April	16 days at 75° F., Humidity .80 to .86			128
3 " 3 "	18 " " "			130
2 " 6 "	21 " " "			133
2 " 8 "	23 " " "			135
1 flea on 11 "	26 " " "			138
3 fleas on 15 "	31 " " "			143
1 flea on 19 "	34 " " "			146
1 " 24 "	39 " " "			151
1 " 16 June	92 " " "			204

Remaining cocoons opened 3 July '12 contained 2 dried up fleas, 1 dried up pupa, 3 dried up larvae, 9 "false" cocoons.

SECOND SERIES. "*Half grown*" larvae of *C. fasciatus* taken from breeding cage and allowed to finish feeding and to spin cocoons in Incubator 75 Wet.

Batch A left in Incubator 75 Wet. Temperature about 75° F. Humidity about 80 to 86.

Date	No. of cocoons	Emergence of fleas	Approximate no. of days in cocoon	No. of days in cocoon calculated from average date given in col. 1
18—26 March '12 (average date 22 March)	31	2 on 1 April '12	say 14	10
		6 3 "	" 16	12
		3 8 "	" 20	17
		5 11 "	" 20	20
		1 16 "	" 25	25
		4 23 "	" 32	32
		1 27 "	" 36	36
		1 30 "	" 35	39
		1 6 May '12	" 41	45
		2 16 "	" 51	55
		2 21 "	" 56	60
		1 24 "	" 59	63
		1 28 "	" 63	67

Cocoons opened 4 July '12, 1 "false" cocoon.

Batch B transferred to Incubator 93 Wet. Temperature about 93° F. Humidity about 67.

Date	Number of cocoons	Emergence of fleas	No. of days in cocoon
18—26 March '12	32	2 on 28 March '12	say 10

Cocoons opened 2 July '12 contained 22 dried up fleas, 7 dried up larvae, 1 "false" cocoon.

SECOND SERIES. "*Half grown*" larvae of *C. fasciatus* allowed to finish feeding and to spin cocoons in the breeding cage.

Batch C left in Cage.

Date	No. of cocoons	Emergence of fleas	Approximate no. of days in cocoon	No. of days in cocoon calculated from average date given in col. 1
18 April—	18	4 on 24 May	say 36	30
8 May '12	2	20 June	" 63	57
(average date 24 April)	1	3 July	" 76	Cocoon opened and living flea emerged 70
	2	10 Aug.	" 114	118
	2	13 "	" 117	121
	1	1 Sept.	" 127	130
	*2	9 "	" 135	Cocoons opened and living fleas emerged 138

10 cocoons opened 9 Sept. '12 contained 1 dead flea, 2 living fleas, 2 dead larvae, 1 living larva which subsequently died.

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Batch D transferred to Incubator 93 Wet. Temperature about 93 to 95° F.
Humidity about .70 to .75.

Date	Number of cocoons	Emergence of fleas	No. of days in cocoon
18 April—2 May '12	18	none	—

Cocoons opened 2 July '12 contained 2 dried up fleas, 4 dried up pupae, 4 resting larvae.

The resting larvae were transferred to Incubator 75 Wet to see if they would continue their development, they died without becoming pupae.

SECOND SERIES. "*Full grown*" larvae of *C. fasciatus* taken from breeding cage and allowed to spin cocoons in Incubator 75 Wet.

Batch A left in Incubator 75 Wet. Temperature about 75° F. Humidity about .80 to .85.

Date	No. of cocoons	Emergence of fleas	Approximate no. of days in cocoon	No. of days in cocoon calculated from average date given in col. 1
14—19 March '12	33	1 on 22 March	say 8	6
(average date		9 26 "	" 12	10
16 March)		6 29 "	" 15	13
		4 1 April	" 18	16
		2 21 May	" 63	66
		1 24 "	" 66	69
		1 3 June	" 76	79
		1 8 "	" 81	85

Cocoons opened 4 July '12 contained 5 dried up larvae.

Batch B transferred to Incubator 93 Wet. Temperature about 93° F.
Humidity about .67 to .70.

Date	No. of cocoons	Emergence of fleas	Approximate no. of days in cocoon	No. of days in cocoon calculated from average date given in col. 1
14—19 March '12	33	1 on 22 March	say 8	6
(average date		6 26 "	" 12	10
16 March)		1 28 "	" 14	12
		2 1 April	" 17	16
		1 8 "	" 24	23
		2 12 "	" 28	27

Cocoons opened 2 July '12 contained 7 dried up fleas, 11 dried up larvae.

SECOND SERIES. "*Full grown*" larvae of *C. fasciatus* allowed to spin their cocoons in the breeding cage.

Batch C left in Cage.

Date	No. of cocoons	Emergence of fleas	Approximate no. of days in cocoon	No. of days in cocoon calculated from average date given in col. 1
19 March—	21	2 on 23 April	say 35	28
8 April '12		3 27 "	" 39	32
(average date		2 2 May	" 38	37
26 March)		1 1 July	" 98	97
		1 3 "	" 100 Cocoon opened	99
		1 8 Aug.	" 136	135
		1 9 Sept.	" 168	167
		1 10 Oct.	" 199	198
		2 26 "	" 213	214

Remaining cocoons opened 25 July '13, one contained a living larva which had been resting between 473 and 493 days, remaining cocoons empty.

Batch D transferred to Incubator 93 Wet. Temperature about 93° F.
Humidity about 70 to 75.

Date	Number of cocoons	Emergence of fleas	Approximate no. of days in cocoon
19 March—8 April '12	21	1 on 18 April	say 30
		1 5 Aug.	„ 96

Cocoons opened 2 July '12 contained 6 dried up fleas, 4 dried up pupae, 9 dried up larvae, 1 resting larva. The resting larva was transferred to Incubator 75 Wet, 2 July. This larva pupated on 30 July. A flea emerged on 5 Aug., say 90 days in Incubator 93 Wet. This individual therefore continued resting without a cocoon in Incubator 75 Wet for 28 days and emerged as adult in 6 days. Total 96 days.

iv. Significance of "Hard" and "Soft" cocoons of *C. fasciatus*.

Experiments were made in order to ascertain whether those individuals that formed hard cocoons deferred emergence for a longer period than those whose cocoons were of the softer type. Two sets of experiments were performed, one in which sand was used and one in which house dust replaced the sand to test whether the nature of the attached fragments had any influence upon the character of the cocoon.

When the larvae of *C. fasciatus* are given finely sifted house dust (sweepings of floors) in place of silver sand the silk of their cocoons differs from that of those formed in sand. The cocoons are tougher and the silk is whiter and more papery in appearance, and is less suggestive of thin glue that has been allowed to harden, than is the case when grains of sand are embedded in place of dust.

It seems possible that this variation is in part, perhaps largely, due to the small size of the particles of dust and their more absorbent nature producing a different action in the setting of the silk. Sand is composed of larger particles which are individually non-absorbent, and this no doubt delays the drying and affords an opportunity for the threads of mucilage to coalesce into larger masses before drying takes place¹.

¹ My assistant Mr J. H. Turner states that he has noticed that the "hard" cocoons generally have smaller grains of sand attached than the "soft"; presumably such a difference must rest on a selective act on the part of the larvae when spinning their cocoons. There is also a well marked difference between the individual cocoons of *C. fasciatus* apart from the material worked into them, and although I have not opened sufficient examples to make any definite generalisation, the cocoons of the different species show marked divergencies when all are spun in sand. Cocoons of *X. cheopis* are softer as well as tougher than those of *C. fasciatus*, approximating towards the type of *P. irritans* which is composed of a felt of actual silk threads with the extraneous matter attached on the outer side.

A number of larvae of *C. fasciatus* were taken from the cages and allowed to spin in incubator 75 Wet, in the case of one series in sand and of the other in dust. The stronger cocoons among those spun in dust are more correctly described as "firm" than "hard"; for the sake of uniformity, however, the latter term has been used in both series of experiments. The question as to whether an individual cocoon should be regarded as "soft" or "hard" was decided by pressing upon the cocoon with the side of a fine needle, near the point. Those which yielded were designated as "soft," those which resisted as "hard." That the stage of development of the insect has nothing to do with the consistence was shown by the fact that some of the resistant cocoons were found to be empty. When covered with sand the hardness was more extreme, quite considerable pressure being required to crush empty ones.

The cocoons were allowed to remain in incubator 75 Wet for a few days after spinning and were then sorted out into batches of "hard" and "soft" respectively and then transferred to incubator 93 Dry. In the sand series some cocoons of each class were kept in incubator 75 Wet as a control. A few examples of those spun in dust, opened to test the reliability of the sorting, gave the following particulars:

8 "soft" showed	9 "hard" showed
1 pupa	3 pupae
1 resting larva	3 resting larvae
6 empty (fleas emerged)	3 empty (fleas emerged)

While the results obtained support the view that the larvae in "hard" cocoons are better able to resist conditions of heat and drought than those in the "soft," it is not clear that the *solidity* of the cocoon is the only factor. It will be noticed that there was a higher mortality among the "soft" than the "hard" in incubator 75 Wet, sand series, which suggests that "hard" cocoons may be associated with individuals of greater vitality and that the question is not merely one of the type of cocoon or a special adaptation for resting. It is, however, true that the resting stage is longer in the hard cocoons, which favours the theory that the greater solidity of these cocoons diminishes the risks of a deferred emergence.

These experiments also bring into special prominence the inhibitory action of incubator 93 Dry with regard to emergence, which offers a contrast to the results obtained in incubator 75 Wet, where the average duration of the cocoon period is very short in comparison.

"Hard" and "Soft" Cocoons *C. fasciatus*.

Cocoons spun in dust. Larvae taken from cages and allowed to spin their cocoons in finely sifted house dust in Incubator 75 Wet and then transferred to Incubator 93 Dry (humidity about '56 to '60).

"Soft" batch.

Date	Number	Fleas emerged	No. of days in cocoon
6 Nov. '11	22	5 on 21 Nov.	15
		7 25 "	19

On 16 March '12 after 131 days in Incubator 93 Dry, 2 cocoons were opened and 1 resting larva, 1 empty ("false" cocoon), were found.

The remaining cocoons and the resting larva were transferred to Incubator 75 Wet. The larva died. Fleas emerged from cocoons as under :

				Total no. of days in cocoon, including 131 days in Incubator 93 Dry
1 flea emerged 1 April	16 days at 75° F., Humidity '79			147
" " 16 "	31 " " "			162
" " 23 "	38 " " "			169

On 4 July '12 remaining cocoons opened, 5 dried up larvae found.

"Hard" batch.

Date	Number	Fleas emerged	No. of days in cocoon
6 Nov. '11	29	6 on 24 Nov.	18
		1 27 "	21

On 16 March '12, 2 cocoons were opened, both contained resting larvae; these together with the remaining cocoons were transferred to Incubator 75 Wet and there emerged

				Total no. of days in cocoon including 131 days in Incubator 93 Dry
2 fleas emerged 1 April	16 days at 75°F., Humidity '79			147
8 " " 6 "	21 " " "			152
1 flea " 8 "	23 " " "			154
2 fleas " 11 "	26 " " "			157
1 flea " 30 "	45 " " "			176

On 4 July cocoons opened, contained 3 dried up fleas, 3 dried up larvae.

From a few of the unsorted cocoons which were left in Incubator 75 Wet, when the others were transferred to Incubator 93 Dry, fleas emerged as follows :

Date	Number	Fleas emerged	No. of days in cocoon
6 Nov. '11	Number unknown	1 on 21 Dec. '11	45
		2 24 "	48
		1 12 Jan. '12	67
		1 16 "	71
		4 18 "	73

"Hard" and "Soft" Cocoons *C. fasciatus*.

Cocoons spun in sand. Larvae taken from cages and allowed to spin their cocoons in fine silver sand in Incubator 75 Wet and then transferred to Incubator 93 Dry (humidity about .56 to .60).

"Soft" batch.

Date	Number	Fleas emerged	No. of days in cocoon
5 Nov. '11	24	1 on 16 Nov. '11	11

On 16 March '12, 8 cocoons were opened and found to contain 1 dead flea, 4 dead larvae, 3 resting larvae.

The resting larvae and the remaining cocoons were transferred to Incubator 75 Wet. (Two of the larvae died, one pupated and a flea duly emerged.)

4 fleas emerged 6 April, 132 days at 93° F., Humidity .56 to .60; 21 days at 75° F., Humidity .79. Total 153 days.

Remaining cocoons opened 3 July '12 contained 3 dried up fleas, 6 dried up larvae, 2 "false" cocoons.

"Hard" batch.

Date	Number	Fleas emerged	No. of days in cocoon
5 Nov. '11	39	none	—

On 16 March '12, after 132 days in Incubator 93 Dry, 11 cocoons were opened and found to contain 2 dead fleas, 7 larvae somewhat shrunk and devoid of movement but not discoloured or dried, 2 resting larvae which subsequently pupated and produced adults.

The resting larvae and remaining cocoons were transferred to Incubator 75 Wet.

Total number of days in cocoon including 132 days in Incubator 93 Dry

2 fleas emerged 3 April	18 days at 75° F., Humidity .79	150
2 " 6 "	21 " " "	153
4 " 8 "	23 " " "	155
2 " 11 "	26 " " "	158
1 flea emerged 16 "	31 " " "	163
1 " 27 "	42 " " "	174

Cocoons opened 3 July '12 contained 3 dried up fleas, 7 dried up larvae¹.

"Hard" and "Soft" Cocoons *C. fasciatus*.

Cocoons spun in sand. Larvae taken from cages and allowed to spin their cocoons in fine silver sand in Incubator 75 Wet and left under these conditions for the fleas to emerge (remainder of batch of cocoons used for experiments in previous table).

"Soft" batch.

Date	Number	Fleas emerged	No. of days in cocoon
5 Nov. '11	24	1 on 13 Nov. '11	8
		1 16 "	11
		4 18 "	13
		2 19 Dec. '11	44
		3 24 "	49
		1 1 Jan. '12	57

Cocoons opened 3 July '12 contained 8 dried up larvae¹.

¹ The record for "hard" and "soft" cocoons spun in sand is incomplete, no record was made on July 3rd 1912 of the number of empty or "false" cocoons in the "hard" batch in Incubator 93 Dry or in the "soft" batch in Incubator 75 Wet.

"Hard" batch.

Date	Number	Fleas emerged	No. of days in cocoon
5 Nov. '11	39	1 on 13 Nov. '11	8
		1 16 "	11
		2 25 "	20
		1 19 Dec. '11	44
		5 22 "	47
		2 23 "	48
		2 24 "	49
		2 26 "	51
		3 27 "	52
		3 28 "	53
		4 1 Jan. '12	57
		2 4 "	60
		2 8 "	64
		1 9 "	65
		2 11 "	67
		1 29 "	85
		1 11 Feb. '12	98

Cocoons opened 3 July '12, 4 empty, "false" cocoons.

4. ADULTS (Tables XLV to LIX).

i. *Duration of Life unfed.*

P. irritans (Tables XLV and L). The experiments with adult specimens of *P. irritans* just emerged from their cocoons and unfed are too few to be conclusive; the results obtained suggest, however, that the species cannot survive long without feeding if exposed to warmth or heat, even if the conditions are humid. In cool and moist places, however, a life of more than 100 days was recorded (see Table XLV). Allowing them to burrow free in sand does not appear to favour an extended life.

C. fasciatus (Tables XLVI and L) when kept in paper tubes, cannot compete with *P. irritans* as regards its power of surviving without food. When kept in a paper tube no life of more than 31 days was recorded even in the most favourable situation; but, as already noted in the general remarks on life history, when allowed to burrow in sand an individual survived starvation for 95 days. In less favourable situations there is not much to choose between the species.

X. cheopis (Tables XLVII and L). It does not appear that adults of *X. cheopis* differ very much from *C. fasciatus* in their power of enduring starvation. It is possible that they stand hot and dry conditions a little better, but, to judge from the beehive record, they are quite unsuited for a cold situation. Burrowing in sand would appear to increase their powers of resistance.

Ct. canis (Tables XLVIII and L). This species has powers of endurance about equal to those of *C. fasciatus* when tested by the paper tube method; burrowing free in sand does not seem to appreciably extend its powers of survival unfed.

C. gallinae (Tables XLIX and L). Only a small number were available and these were used for tests in cool situations only. When tested by the paper tube method they show endurance on about the same scale as *C. fasciatus* or *X. cheopis*, but burrowing in sand would appear to make a vast difference to them, one specimen surviving 127 days and showing an endurance equalled only by *P. irritans*.

ii. *Duration of Life when fed.*

(a) *for an initial period only.* *P. irritans* and *C. fasciatus* (Tables LI and LII). Newly emerged specimens of these two species were fed for certain periods and then tested as in the previous series. It will be seen from a comparison of Tables XLV and XLVI with LI and LII respectively that, under cool conditions with high humidity, the fact of a flea having previously fed makes little material difference to its powers of endurance, but that under warm to high temperatures with a low humidity, a meal to start with is a very valuable asset.

For example, in case of *P. irritans*, in the cellar, the longest length of life when starved subsequent to a period of feeding was 121 days, while, in case of adults starved from emergence, a life of 126 days was recorded. In incubators 75 Dry and 85 Dry, on the other hand, previous feeding raised the figure obtained for length of life from 5 to 17 and 5 to 12 days respectively.

It is greatly to be regretted that only single individuals of *P. irritans* were available for this test. The consistent increase in length of life recorded as we pass from hot and dry to cool and moist situations may, however, be taken as confirmatory of the trustworthiness of the results.

C. fasciatus. This species, like *P. irritans*, does not gain by feeding prior to starvation; feeding for several days tends to lessen, not increase, their powers of survival and leaves the average total length of life practically unaltered.

Ct. canis (Table LIII). The small trial made with this species cannot be compared with the *P. irritans* and *C. fasciatus* tests, as the specimens were of unknown age, and had already been 24 hours in incubator 75 Wet before the test was made.

(b) *At regular intervals.* *P. irritans.* When fed on man daily, individual specimens of this species have lived for various periods up to 513 days (see Table LIV (c)), and, when fed weekly, also over a year if kept under cool humid conditions (see Table LIV (d)).

It will be seen from Table LIV that the summer is just as favourable as the winter. Several of the early deaths recorded for this species were due to accident, becoming gummed to the glass bottom of the box by their own dejecta or losing portions of the legs. On a few occasions, deaths have been quite inexplicable; in one instance some six or eight freshly emerged specimens all died within 10 or 12 days, although afforded equal opportunities of feeding with batches that enjoyed extended lives.

Buckland records (*Curiosities of Natural History*, fourth series, p. 123) on the authority of the owner of some performing fleas, a specimen which lived for eighteen months—a period which slightly exceeds the longest life recorded in the present experiments.

C. fasciatus. The number and variety of other experiments on hand prevented the carrying through of cage experiments with this species and *X. cheopis* to test the length of life of these species when free upon their natural hosts. Two or three early attempts failed, owing to paucity of supplies, and it has not been found possible to repeat them. Experiments in which the fleas were fed on rats by the box method, point to the necessity of cool, or at least moderate conditions of temperature (see Table LV, under date of 12th October, 1910, in incubator 85 Wet) and, when not living in close association with the rat, to the need of more frequent chances of feeding than were found necessary for *P. irritans*. The longest life recorded is 106 days, and this is probably nearer to the correct length of time than the average lives—the early deaths which bring down the average being probably those of individuals which never commenced feeding.

iii. *Duration of Life if fed on an alien host.*

C. fasciatus fed on man (Table LVI). When fed on man the average length of life is, on the whole, higher than when fed on rats by the box method, possibly because the feeding being conducted at night while in bed the time was apt to be extended longer than the usual 15 minutes. The 10 deaths in 13 days, recorded in the experiment under date 15th July, 1911, were due to a misadventure and should not be considered when judging results.

X. cheopis. The experiment (Table LVI) shows this species to be better adapted to a human host than *C. fasciatus*, lives of over 70 days

being frequent. Observation of the specimens during the course of the experiment provides confirmatory evidence of this, in that they were seen to be very lively and to be feeding vigorously, judging by the full state of their stomachs.

Ct. canis and *Ct. felis*. It will be seen from Table LVII that these two species can exist quite successfully on a diet of human blood—the lives recorded being of considerable extent, as long as 345 and 185 days respectively.

C. gallinae (Table LVII) is also able to live for a considerable period when fed on man. The single specimen captured on the dog lived 224 days after capture.

L. musculi (Table LVII). A certain percentage of individuals of this species will certainly feed on man, the blood was quite visible in previously unfed specimens, but they do not bite freely, as do *X. cheopis* and *C. fasciatus*. Life was only of very short duration and did not exceed 10 days in case of any of the fleas tested.

Spilopsyllus cuniculi (Table LVII). This species feeds much more freely on man than does *L. musculi*; I think all the specimens tried bit, but they are not transparent enough to show blood in their stomachs. As will be seen from the table, their record is consistent with this view.

Attempts to feed *P. irritans* and *Ct. canis* on rats (Table LV) show that the last named species will certainly feed, but, as regards the former, it is somewhat doubtful whether the insects did more than experiment, the lives of those fed upon rats by the box method being no longer than one would expect if kept unfed under the same circumstances.

iv. *Attempts to induce fleas to feed upon material other than the blood of a living animal.*

The experiments in which fleas were given the opportunity of sucking water or feeding on syrup, broth, etc., have not been successful in establishing as a fact whether they do feed, or whether they only obtain a benefit by the moisture in the air obtained by a pad of blotting paper moistened being placed in the box with them. The fleas were observed to jump on the pad and sometimes put down their heads as if to feed, but as the process was only followed with a hand lens and the fleas were kept in a box it is not possible to say whether suction actually took place.

A number of dissections was made of individuals kept under similar conditions to those mentioned above; carmine and other stains,

that were successfully used with larvae of fleas and of flies, were mixed with the artificial foods or water, but no trace of the stain was ever found in the gut of the adult flea.

TABLE XLV. *Adult fleas. P. irritans. Length of life unfed. Fleas freshly emerged from cocoons, placed in filter paper tubes and buried in sand in various situations.*

Date	No.	Place	Temperature		Humidity	Detail	
12 May '11	1	Incubator 85 Wet	83·8		·81	dead in 11 days	
12 „	1	Incubator 75 Wet	75·1		·91	„	9 „
10 „	1	Incubator 85 Dry	84·0		·61	„	5 „
10 „	1	Incubator 75 Dry	74·7		·53	„	5 „
15 „	1	Warm Cupboard	63·5		·65	„	6 „
			max.	min.			
15 „	1	Lab. Cupboard	62·6	54·1	·81	„	11 „
4 Aug. '10	2	„	74·4	66·0	·74	{ 1 „ 9 „	
						{ 1 „ 10 „	
12 Dec. '10	1	Damp corner of Cellar	47·3	46·0	over ·94	„	71 „
1 Mar. '11	1	„	51·8	50·3	„ ·93	dead in 126—130 days	
27 April '11	2	„	57·6	56·3	„ ·93	{ 1 dead in 63 days	
						{ 1 „ 91 „	
18 Aug. '11	1	„	62·9	61·6	„ ·94	escaped after 1 month	
19 „	3	„	62·2	61·3	„ ·94	{ 1 dead in 28 days	
						{ 2 „ 32 „	
28 April '11	2	Beehive	69·5	43·1	—	{ 1 „ 15 „	
						{ 1 „ 20 „	

TABLE XLVI. *Adult fleas. C. fasciatus. Length of life unfed.*

(a) *Fleas freshly emerged from cocoons, placed in filter paper tubes and replaced in soil pots in the situations in which they had been reared from cocoons.*

Dates between which the fleas were obtained	No.	Place	Temperature	Humidity	Detail	Longest period of survival, days	Average period of survival, days
17 Aug. '10—	18	Incubator	Dry 84·5	·71	2 dead in 4 days	13	8
28 Sept. '10		85 Wet			1 „ 6 „		
					8 „ 7 „		
					1 „ 8 „		
					1 „ 9 „		
					1 „ 11 „		
					1 „ 12 „		
					2 „ 13 „		
					1 lost		

TABLE XLVI.—*Continued.*

Dates between which the fleas were obtained	No.	Place	Temperature	Humidity	Detail	Longest period of survival, days	Average period of survival, days
15 Aug. '10—	13	Incubator	Dry 74·3	·65	4 dead in 5 days	7	6
29 „		75 Wet			3 „ 6 „		
					6 „ 7 „		
19 Aug. '10—	17	Laboratory	estimate 60·0	·80	2 „ 5 „	15	10
3 Oct. '10		Cupboard			1 „ 6 „		
					7 „ 10 „		
					1 „ 11 „		
					1 „ 12 „		
					2 „ 13 „		
					2 „ 14 „		
					1 „ 15 „		
25 Aug. '10—	13	Cellar	max. min. 58·3 57·3	estimate ·90	2 „ 7 „	17	14
6 Oct. '10					1 „ 10 „		
					2 „ 12 „		
					1 „ 13 „		
					1 „ 15 „		
					1 „ 16 „		
					5 „ 17 „		
1—7 Dec. '10	1	„	48·7 47·7	·93	1 „ 6 „	—	—
(b) <i>Fleas taken from stock reared in Incubator 75 Wet.</i>							
28 Dec. '10—	12	Cellar	46·5 45·3	·93	2 „ 17 „	31	24
28 Jan. '11					4 „ 21 „		
					4 „ 28 „		
					2 „ 31 „		
28 Dec. '10—	7	Laboratory	53·5 43·2	·85	1 „ 10 „	20	16
17 Jan. '11		Cupboard			2 „ 14 „		
					2 „ 17 „		
					2 „ 20 „		
7 Jan. '11—	10	Beehive	44·3 32·2	—	2 „ 10 „	24	15
31 „					3 „ 13 „		
					3 „ 17 „		
					1 „ 21 „		
					1 „ 24 „		
12 Sept. '11	4	Incubator	Dry 75·0	·54	1 „ 3 „	5	4
		75 Dry			2 „ 4 „		
					1 „ 5 „		
12 „	4	Incubator	Dry 84·1	·59	4 „ 3 „	—	—
		85 Dry					

TABLE XLVII. *Adult fleas. X. cheopis. Length of life unfed. Fleas freshly emerged from cocoons, spun in Incubator 75 Wet, placed in filter paper tubes and buried in sand in various situations.*

Date 1911	Number	Place	Temperature max. min.	Humidity	Detail	Longest period of survival, days	Average period of survival, days
7 Oct.	6	Cellar	56·0 54·4	·93	3 dead in 23 days	28	25
					2 „ 27 „		
					1 „ 28 „		
7 „	6	Incubator 75 Wet	74·2	·72	2 „ 7 „	13	10
					1 „ 9 „		
					2 „ 12 „		
					1 „ 13 „		
19 „	10	„ 75 Dry	74·8	·56	5 „ 4 „	6	5
					4 „ 5 „		
					1 „ 6 „		
19 „	10	„ 85 Dry	84·0	·61	1 „ 2 „	5	4
					6 „ 4 „		
					3 „ 5 „		
19 „	10	„ 85 Wet	84·2	·80	1 „ 6 „	13	9
					3 „ 8 „		
					3 „ 9 „		
					2 „ 11 „		
					1 „ 13 „		
19 „	10	Lab. Cupboard	52·5 49·8	·86	2 „ 6 „	17	12
					4 „ 12 „		
					1 „ 14 „		
					3 „ 17 „		
24 „	10	Warm Cupboard	Dry 67·0	·55	4 „ 4 „	6	5
					6 „ 6 „		
24 „	10	Beehive	56·2 34·5	—	3 „ 4 „	6	5
					7 „ 6 „		

TABLE XLVIII. *Adult fleas. Ct. canis. Length of life unfed. Fleas, freshly emerged from cocoons, reared in Incubator 75° Wet, from larvae taken from the dog's bed. The fleas were placed in filter paper tubes and buried in sand in various situations.*

Date of emergence and commencement of test	No.	Place	Temperature	Humidity	Detail	Longest period of survival, days	Average period of survival, days
7 Nov. '10	5	Incubator 85° Wet	84·0	·71	5 dead in 6 days	—	—
17 "	9	"	84·0	·70	4 " 4 " 5 " 8 "	8	6
17 "	8	Incubator 75° Wet	74·3	·73	1 " 5 " 2 " 8 " 4 " 11 " 1 " 13 "	13	9
28 "	13	Warm Cupboard	59·8	·60	3 " 4 " 10 " 6 "	6	5
7 "	5	Cellar (moist corner)	max. 45·0 min. 43·7	over ·93	1 " 12 " 3 " 21 " 1 " 32 "	32	21
13 Dec. '10	10	Box (out of doors)	No record, there were several sharp frosts	—	4 " 6 " 3 " 10 " 2 " 25 " 1 " 29 "	29	13
10 Nov. '10	4	Laboratory Cupboard	54·0 42·8	about ·84	2 " 4 " 1 " 9 " 1 " 11 "	11	6
17 Aug. '11	4	Cellar (moist corner)	Aug. 64·5 63·4 Sept. 60·6 59·5	over ·94 " ·93	2 " 34 " 2 " 44 "	44	39
21 Sept. '11	10	Incubator 75° Dry	74·7	·51	4 " 4 " 4 " 5 " 1 " 6 " 1 " 7 "	7	5
21 "	10	Incubator 75° Wet	74·4	·73	2 " 5 " 6 " 9 " 2 " 10 "	10	8
21 "	10	Incubator 85° Dry	84·6	·56	1 " 2 " 6 " 4 " 3 " 5 "	5	4
21 "	10	Incubator 85° Wet	84·7	·77	2 " 5 " 4 " 6 " 4 " 8 "	8	7

TABLE XLVIII—Continued.

Date of emergence and commencement of test	No.	Place	Temperature	Humidity	Detail	Longest period of survival, days	Average period of survival, days
21 Sept. '11	10	Warm Cupboard	64.2	.66	1 dead in 5 days	10	7
					4 " 6 "		
					3 " 7 "		
					1 " 9 "		
					1 " 10 "		
21 "	10	Beehive	67.4 41.4	—	3 " 9 "	20	14
					3 " 12 "		
					1 " 15 "		
					2 " 19 "		
					1 " 20 "		
22 "	10	Laboratory Cupboard	59.7 51.1	.82	2 " 8 "	24	14
					1 " 11 "		
					2 " 12 "		
					2 " 13 "		
					1 " 17 "		
					1 " 21 "		
					1 " 24 "		
22 "	30	Cellar	Sept. 60.6 59.5	.93	13 " 31 "	57	35
			Oct. 56.3 54.8	.92	14 " 42 "		
			Nov. 50.7 48.9	.91	1 " 52 "		
					1 " 54 "		
					1 " 57 "		
			Put into 1½" ento. box and buried in sand.				
21 "	13	Cellar	Sept. 60.6 59.5	over .93	2 " 15 "	39	31
		(moist corner)	Oct. 56.3 54.8	„ .92	8 " 32 "		
					3 " 39 "		

TABLE XLIX. *Adult fleas. C. gallinae. Length of life unfed. Fleas (apparently freshly emerged from cocoon) obtained from empty nest of blue tit, placed in filter paper tubes and kept in sand pots in various situations.*

Date of emergence and commencement of test	No.	Place	Temperature max. min.	Humidity	Detail	Longest period of survival, days	Average period of survival, days
13 Feb. '11	4	Beehive	51.7 32.7	—	2 dead in 21 days	27	26
					1 " 25 "		
					1 " 27 "		
„	3	Lab. Cupboard	55.3 45.6	.84	1 " 14 "	28	20
					1 " 19 "		
					1 " 28 "		
„	6	Cellar	46.9 45.3	.92	1 " 5 "	45	32
					1 " 35 "		
					1 " 39 "		
					2 " 45 "		
					1 escaped		

TABLE L. *Adult fleas of various species, length of life unfed. Fleas freshly emerged from cocoons spun in Incubator 75 Wet and kept in the cellar in card jars containing sand for them to burrow in.*

Species <i>P. irritans</i>	Date of emergence and commencement of the test 20 Dec. '11	Number 12	Place Cellar	Temperature max. min.	Humidity	Detail Only one active seen after end of January	Longest period of survival 84 days
<i>Ct. canis</i>	4 Jan. '12	4	"	Sept. 60.6 59.5 Oct. 56.3 54.8 Nov. 50.7 48.9	.93 .92 .91	More than one seen active at end of Feb., all dead by 2 March	58 "
<i>X. cheopis</i>	1 Jan. '12	20	"	Dec. 48.7 47.4 Jan. 47.2 45.9 Feb. 45.8 44.6	.92 .92 .92	Several seen active on 1 Feb., all dead by 7 Feb.	38 "
<i>C. gallinae</i>	1-5 Feb. '12	4	"	Mar. 49.1 48.1 Apr. 50.3 49.1 May 54.2 53.2	.93 .93 .93	3 died between 29 May and 1 June, last died 11 June	127 "
<i>C. fasciatus</i>	5 Sept. '11	20 to 30	"	June 56.4 55.6	.93	A number were seen active on 2 Dec. but by the 7th only one feeble in- dividual was to be seen, all were dead by 12 Dec.	about 95 days

TABLE LI. *Adult fleas. P. irritans. Length of life unfed after feeding for a period. Fleas freshly emerged were fed for at least one week on human blood and then kept without food in filter paper tubes placed in sand pots in various situations.*

Date of commencement of test	No.	Place	Temperature max. min.	Humidity	Total length of life, — days	Length of life unfed, days
30 Sept. '10	1	Cellar (moist corner)	50·4 49·2	over ·93	121	112
9 Jan. '11	1	Cellar	46·0 44·6	·92	44	37
27 Feb.	1	„	52·3 50·8	·92	130	120
9 Jan.	1	Beehive	44·2 34·8	—	30	23
27 Feb.	1	„	52·2 32·9	—	45	38
9 Jan.	1	Lab. Cupboard	52·4 43·0	·84	44	37
27 Feb.	1	„	54·9 45·3	·85	31	24
9 Jan.	1	Incubator 75 Wet	74·5	·75	23	16
„	1	„ 85 Wet	84·9	·68	21	14
„	1	„ 75 Dry	76·5	·55	17	10
„	1	„ 85 Dry	85·5	·56	12	5

TABLE LII. *Adult fleas. C. fasciatus. Length of life without further food after feeding on rats' blood for varying periods. A special cage was stocked on 28 Dec. '10, with fleas freshly emerged from cocoons kept in Incubator 75 Wet; these fleas were allowed opportunity of feeding upon a rat for 24 hours and upwards, afterwards being kept without food in filter paper tubes placed in sand pots.*

(a) *In Cellar.*

Date of commencement of test	No.	Allowed access to rat for	Temperature max. min.	Humidity	Details	Longest period of survival unfed, days	Average period of survival unfed, days
29 Dec. '10	18	24 hours	46·5 45·3	·93	2 dead in 17 days	33	26
					4 „ 21 „		
					8 „ 27 „		
					1 „ 30 „		
					3 „ 33 „		
31 Dec. '10	15	48 „	46·5 45·3	·93	2 „ 14 „	32	19
					2 „ 19 „		
					3 „ 25 „		
					2 „ 28 „		
					4 „ 31 „		
					2 „ 32 „		
3 Jan. '11	10	72 „	46·5 45·3	·93	2 „ 16 „	30	24
					2 „ 22 „		
					2 „ 25 „		
					2 „ 28 „		
					1 „ 29 „		
					1 „ 30 „		
7 Jan. '11	11	1 week	46·5 45·3	·93	3 „ 18 „	26	21
					5 „ 21 „		
					2 „ 25 „		
					1 „ 26 „		

TABLE LII—Continued.

(b) In Beehive.

Date of commencement of test	No.	Allowed access to rat for	Temperature max.	min.	Humidity	Details	Longest period of survival unfed, days	Average period of survival unfed, days
9 Jan. '11	10	9 days	43·5	33·0	—	1 dead in 8 days	19	13
						4 " 11 "		
						4 " 15 "		
						1 " 19 "		

NOTE. Allowance must be made for the fact that the natural death rate might have already eliminated weaklings from the cage before the individuals were taken for the later experiments.

TABLE LIII. *Adult fleas. Ct. canis. Length of life without food. Fleas taken from the dog, put into Incubator 75° Wet at 75° F., Humidity .77 for 24 hours (in order to obtain eggs for experiment) and then kept in filter paper tubes buried in sand pots in various situations.*

Date of commencement of test	No.	Place	Temperature max.	min.	Humidity	Detail	Longest period of survival unfed, days	Average period of survival unfed, days
8 Sept. '11	2	Cellar	63	62	.94	all dead in 4 days		
"	2	Lab. Cupboard	73	61	.75	all " 2 "		
"	2	Beehive	87	54·3	—	1 " 1 day	3	2
						1 " 3 days		
"	2	Warm Cupboard	69·0		.66	all " 2 "	—	—
"	2	Incubator 85° Wet	84·6		.81	1 " 1 day	3	2
						1 " 3 days		
"	2	" 75° Wet	75·0		.79	all " 1 day	—	—
"	2	" 75° Dry	75·5		.57	1 " 1 "	2	—
						1 " 2 days		
"	2	" 85° Dry	84·0		.62	all " 2 "	—	—

TABLE LIV. *Adult fleas. P. irritans. Length of life when fed on man.*

(a) *Freshly emerged fleas, kept in gauze-covered boxes in Laboratory or Bedroom and fed daily.*

Date of emergence	No.	Origin		Length of life approximate*	Remarks
		Situation and food during larval stage	Days in cocoon		
30 Mar. '11	1	{ Lab. blanket } { Cupboard, shakings }	about 211	1 dead about 148 days	Commenced to lay 1 May '11
1 April	1	" B.S. Rag	" 160	1 " 160 "	
10 "	1	" Flea faeces	" 228	1 " 161 "	
18 "	1	" B.S. Rag	" 200	1 " 153 "	
21 "	1	" "	" 203	1 " 250 "	
22 "	1	" "	" 215	1 " 251 "	
27 "	1	" "	" 220	1 " 316 "	

* Those which were first to emerge are considered to be the first to die.

TABLE LIV—Continued.

(b) *Freshly emerged fleas, kept in gauze-covered boxes in Laboratory Cupboard and fed twice weekly.*

Date of emergence	No.	Temperature max. min.	Humidity	Length of life approximate
22 Dec. '10	2	Jan. 53·5 44·0	·84	1 dead in 75 days*
24 „	1	Feb. 52·8 43·6	·83	1 „ 204 „
25 „	1	Mar. 55·2 45·5	·85	2 „ 226 „
27 „	2	April 58·2 48·2	·78	1 „ 231 „
		May 61·8 54·3	·78	1 „ 239 „
		June 66·4 58·3	·79	
		July 71·5 62·7	·75	
		Aug. 72·0 64·1	·74	

(c) *Freshly emerged or captured fleas, kept in gauze-covered boxes in Laboratory or Bedroom and fed daily.*

Date of emergence or capture	No.	Origin	Length of life	Sex	Longest period of survival, days	Average period of survival, days
July '10	2	Captured	1 dead in 100 days	?	162	130
			1 „ 162 „	?		
Aug. '10	4	Bred in Lab. Cupboard freshly emerged	1 „ 131 „	?	142	135
			1 „ 142 „	?		
			1 „ 135 „	♂		
			1 „ 135 „	♂		
3 Oct. '10	1	Captured	1 „ 58 „	?	—	—
21 Feb. '11	18	Bred in Lab. Cupboard, freshly emerged (larvae taken from dog's bed Oct. '10)	1 „ 148 „	—	513	348
			1 „ 160 „			
			2 „ 177 „			
			1 „ 190 „			
			1 „ 360 „			
			2 „ 377 „			
			2 „ 388 „			
			1 „ 397 „			
			1 „ 424 „			
			2 „ 434 „			
			1 „ 437 „			
			2 „ 443 „			
			1 „ 513 „			

* This flea was found dead gummed to glass of the box by blood voided by the fleas; it is possible but not definite that this was the cause or contributory cause of death.

TABLE LIV (c).—*Continued.*

Date of emergence or capture	No.	Origin	Length of life	Sex	Longest period of survival, days	Average period of survival, days
3 June '11	10	Bred in Cellar, freshly emerged (larvae taken from dog's bed Oct. '10)	1 dead in 49 days	—	269	126
			1 " 72 "			
			2 " 80 "			
			2 " 84 "			
			1 " 100 "			
			1 " 192 "			
			1 " 258 "			
			1 " 269 "			
20 June '11	17	Bred in Incubator 75 Wet freshly emerged	1 " 58 "	—	252	112
			6 " 67 "			
			4 " 87 "			
			2 " 99 "			
			1 " 203 "			
			1 " 206 "			
			1 " 241 "			
			1 " 252 "			
Fed twice a week.						
9 Mar. '11	7	Freshly emerged	4 " 92 "	—	166	121
			1 " 153 "			
			1 " 158 "			
			1 " 166 "			
(d) <i>Kept in the Cellar and fed daily until 2nd October and then once a week.</i>						
23 Aug. '11	11	Freshly emerged	1 " 103 "	—	511	281
			1 " 192 "			
			2 " 200 "			
			1 " 206 "			
			1 " 278 "			
			2 " 345 "			
			1 " 414 "			
			1 " 505 "			
			1 " 511 "			

TABLE LV. *Adult fleas. Length of life of various species when fed on rats. Fleas freshly emerged from cocoons in Incubator 75 Wet and kept in gauze-covered boxes in various situations.*

Species	Method of feeding	Date of emergence	Number	Place	Temperature max. min.	Humidity	Length of life 2 dead in 24 days	Aver. 28 days
<i>C. fasciatus</i>	Box method, 15 minutes daily	7 Oct. '10	5	Rat's cage	—	—	3 " 31 "	28 "
"	"	7 Feb. '11	10	Lab. Cupboard	56·7 47·7	·81	1 " 8 " 1 " 9 " 5 " 12 " 1 " 14 " 1 " 90 " 1 " 106 "	
"	Box method, 15 minutes every other day	12 Oct. '10	9	Incubator 85 Wet	83·9	·71	1 " 4 " 1 " 10 " 4 " 12 " 2 " 18 " 1 " 20 "	13 "
<i>P. irritans</i>	Box method, 15 minutes daily	4 Feb. '11	6	Lab. Cupboard	52·0 43·7	·84	1 " 3 " 1 " 6 " 1 " 8 " 1 " 9 " 1 " 11 " 1 " 14 "	8 "
"	In cage with rat	30 Nov. '10	6	—	—	—	The last living specimen was seen 40 days from start	
<i>Ct. canis</i>	Box method, 15 minutes daily	17 Nov. '10	10	Lab. Cupboard	58·4 45·4	·85	1 dead in 24 days 1 " 30 " 3 " 34 " 1 " 46 " 1 " 48 " 2 " 54 " 1 " 58 "	41 days
"	In cage with rat	27 Nov. '10	60	—	—	—	No record of individual deaths; they died off gradually, all were dead by 25 Dec. '10	

TABLE LVI. *Adult fleas, C. fasciatus and X. cheopis. Length of life when fed on man. Fleas freshly emerged from cocoons in Incubator 75 Wet, fed daily and kept in Laboratory Cupboard during the day and in Bedroom at night.*

Species	Date of emergence	Number	Length of life		Longest period of survival, days	Average period of survival, days
<i>C. fasciatus</i>	3 Oct. '10	6	1	dead in 17 days	79	39
			2	" 19 "		
			1	" 22 "		
			2	" 79 "		
"	18 Oct. '10	4	1	" 4 "	76	35
			1	" 19 "		
			1	" 41 "		
			1	" 76 "		
"	15 July '11	12	*10	" 13 "	62	18
			1	" 26 "		
			1	" 62 "		
<i>X. cheopis.</i>	15 July '11	19	3	" 18 "	100	38
			†12	" 33 "		
			1	" 55 "		
			1	" 65 "		
			1	" 67 "		
			1	" 100 "		
			approximate			
"	8 June '11	2	1	" 55 "	87	73
	13 "	4	2	" 66 "		
	11 July '11	9	4	" 69 "		
			1	" 70 "		
			3	" 71 "		
			4	" 87 "		

* 10 all died at one time, cause of death unexplained, possibly due to entanglement of legs in piece of cloth put into box to induce females to lay.

† All died on the same date. When removed from the box they were found to have their legs entangled in the cloth; whether this had any relation to their death or if it occurred subsequently to death is not known.

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Species	Date of emergence or capture	Feeding	Origin	No.	Length of life	survival, days	survival, days	Remarks
		Box method, 15 minutes daily	Captured on Dog		1 dead in 38 days			
<i>Ct. canis</i>	30 Oct. '10			3	1 " 52 "	62	51	—
"	7 Nov. '10	"	Bred (freshly emerged)	6	1 " 62 "			—
					2 " 15 "	245	72	
					1 " 31 "			
					1 " 45 "			
					1 " 84 "			
					1 " 245 "			
					2 " 66 "	345	145	—
	17 May '11	"	"	8	2 " 84 "			
					1 " 89 "			
					1 " 156 "			
					1 " 275 "			
					1 " 345 "			
<i>Ct. felis</i>	29 Dec. '10	"	"	5	1 " 139 "	185	156	—
					1 " 146 "			
					1 " 152 "			
					1 " 157 "			
					1 " 185 "			
<i>C. gallinae</i>	7 Nov. '10	"	Captured on Dog	1	1 " 224 "	—	—	This specimen was a female, she laid a few infertile eggs during the spring of 1911
"	12 Feb. '10		Captured in nest of Blue Tit (apparently freshly emerged)	7	1 " 6 "	41	18	These fleas were observed to copulate before feeding commenced. *One female laid fertile eggs after some weeks captivity. 11 fleas were reared from these eggs
					2 " 8 "			
					1 " 15 "			
					1 " 22 "			
					1 " 25 "			
					*1 " 41 "			
<i>L. musculi</i>	31 May '12	15 to 20 minutes daily	Bred in Incubator 75° Wet, larvae taken from cages (freshly emerged)	11	5 " 4 "	10	6	One noted very full of blood
					2 " 5 "			
					3 " 8 "			
					1 " 10 "			
	19 April '12	"	"	6	2 " 3 "	5	4	"
					3 " 4 "			
					1 " 5 "			
<i>Spilopsyllus cuniculi</i>	12 April '12	"	Reared from a rabbits' nest	19	9 " 13 "	69	27	Kept under living room conditions when not feeding
					3 " 24 "			
					4 " 38 "			
					1 " 52 "			
					1 " 56 "			
					1 " 69 "			

TABLE LVIII. *Adult fleas. Ct. canis and C. fasciatus. Length of life without food when kept in various situations and supplied with a small piece of blotting paper moistened with distilled water each night.*

Species	Date of emergence or capture	Origin	No.	Place kept during test	Temperature max. min.	Humidity	Longest period of survival, days		Average period of survival, days
							1 dead in 9 days	Detail	
<i>Ct. fasciatus</i>	18 Nov. '10	Bred, freshly emerged	4	Lab. Cupboard	55.0 45.6	.85	1	12 "	27
							1	" 39 "	
							1	" 48 "	
	4 "	Captured	4	"	54.0 42.8	.84	1	" 21 "	23
							2	" 24 "	
							1	" 26 "	
<i>Ct. canis</i>	17 "	Bred, freshly emerged	10	Incubator 85 Wet	Dry 83.9	.71	1	" 4 "	8
							5	" 8 "	
							4	" 10 "	
	17 "	"	10	" 75 Wet	74.3	.73	1	" 4 "	10
							3	" 10 "	
							1	" 12 "	
							1	" 15 "	
	17 "	"	9	Warm Cupboard	59.5	.57	1	" 11 "	17
							2	" 14 "	
							3	" 17 "	
							1	" 18 "	
							1	" 20 "	
							1	" 21 "	
	17 "	"	8	Lab. Cupboard	55.3 46.2	.85	1	" 11 "	20
							2	" 15 "	
							1	" 18 "	
							2	" 22 "	
							1	" 27 "	
							1	" 34 "	
	17 Feb. '11	"	13	Incubator 75 Dry	75.6	.51	1	" 3 "	6
							6	" 4 "	
							2	" 5 "	
							1	" 7 "	
							1	" 9 "	
							2	" 10 "	
							4	" 3 "	5
	21 "	"	10	" 85 Dry	83.9	.59	4	" 6 "	
							1	" 9 "	
							1	" 10 "	

(Remainder escaped)

TABLE LIX. *Adult fleas. Ct. canis and C. fasciatus. Length of life of freshly emerged specimens, when supplied with various artificial foods. Liquid food supplied on pad of blotting paper or greasy sheeps' wool put into their boxes and remoistened each night.*

Species	Nature of liquid supplied	Date of emergence	No.	Place kept during test	Temperature max. min.	Humidity	Length of life dead in 4 days	Longest period of survival, days	Average period of survival, days
<i>Ct. canis</i>	(A) Broth on blotting paper	17 Aug. '11	10	Lab. Cupboard	70.5 61.8	.76	5	22	8
							1 "	5 "	
							1 "	7 "	
							1 "	12 "	
							1 "	18 "	
							1 "	22 "	
	(B) Water on blotting paper	15 Aug.	14	"	70.3 61.8	.75	1 "	6 "	13
							2 "	7 "	
							3 "	9 "	
							1 "	12 "	
							1 "	15 "	
							3 "	16 "	
							1 "	18 "	
							1 "	20 "	
							1 "	26 "	
<i>C. fasciatus</i>	(A) Broth with sugar on blotting paper	26 May	10	"	68.8 61.4	.76	1 "	4 "	6
							3 "	5 "	
							1 "	6 "	
							5 "	8 "	
	(B) Water on blotting paper	26 May	10	"	66.6 58.9	.79	1 "	12 "	22
							1 "	14 "	
							1 "	16 "	
							1 "	17 "	
							1 "	22 "	
							2 "	23 "	
							1 "	29 "	
							1 "	42 "	
							1 escaped		

TABLE LIX.—Continued.

Species	Nature of liquid supplied	Date of emergence	No.	Place kept during test	Temperature max. min.	Humidity	Length of life	Longest period of survival, days	Average period of survival, days
<i>C. fasciatus</i>	(A) Broth with sugar on blotting paper	29 June	12	Lab. Cupboard	68·1 60·1	·80	6 dead in 8 days	12	9
							5 "	10 "	
							1 "	12 "	
"	(A) Broth with sugar on pad of wool	29 May	8	Incubator 75 Wet	74·2	·91	1 " 1 day	2	2
							7 "	2 days	
"	(B) Water on pad of wool	30 May	10	"	75·9	·80	1 " 1 day	6	4
							2 " 3 days		
							1 "	4 "	
							5 " 6 "		
							1 escaped		
"	(A) Broth with sugar on pad of wool	1 June	11	Cellar	59·2 57·7	·93	1 dead in 2 "	13	7
							2 " 4 "		
							3 " 5 "		
							2 " 7 "		
							3 " 13 "		
"	(B) Water on pad of wool	2 June	10	"	58·8 57·2	·93	2 " 6 "	13	11
							6 " 12 "		
							2 " 13 "		
"	(A) Broth on blotting paper	10 July	9	Lab. Cupboard	70·6 60·8	·74	1 " 3 "	6	5
							7 " 5 "		
							1 " 6 "		
"	(B) Water on blotting paper	10 July	7	"	72·9 63·7	·73	1 " 11 "	21	17
							2 " 16 "		
							2 " 21 "		
							2 escaped		
"	(B) " " "	15 Aug.	35	Incubator 75 Dry	74·7	·59	2 dead in 4 "	15	8
							3 " 6 "		
							8 " 7 "		
							7 " 8 "		
							6 " 9 "		
							2 " 10 "		
							5 " 11 "		
							1 " 13 "		
							1 " 15 "		

v. *Possibility of continued breeding in the absence of any host.*

Some experiments were made in order to see whether fleas could establish themselves in the absence of a host, by throwing successive broods, the necessary nutriment for reproduction by the adults being obtained, as in case of so many insects, by the surplus passed on from the larval period. Many of the experiments with adults, although performed for other purposes, afford evidence bearing on this subject, but the procedure was arranged in these special tests with a view to making the attendant circumstances as favourable as possible. The evidence obtained is entirely negative in character.

P. irritans. A large glass jar was stocked with a quantity of carpet sweepings and greasy unwashed sheep's wool. Some 270 newly hatched larvae were added and the jar was placed in the cellar on the 22nd May, 1911. Periodic examinations were made from 23rd September, 1911, onwards to September, 1912. A few adult fleas were seen during September and October, 1911, but none afterwards and no trace of larvae could be found.

C. fasciatus. A similar glass jar, stocked in the same way, was placed in the cellar on the 30th May, 1911, and 110 newly hatched larvae of *C. fasciatus* were added. A number of adult fleas (36 in all) were found from time to time up to the 10th May, 1912. Subsequent examinations up to September, 1912, were barren and no trace of any larvae could be found.

C. gallinae. Material taken from a nesting box used by a blue tit was received in June, 1911. It was kept in a glass jar in the beehive. Neither larvae nor fleas could be found when it was taken from the box. An examination in September, 1911, showed that some *C. gallinae* had emerged; cocoons were also discovered and opened and found to contain living fleas. Fleas continued to emerge up to the 24th April, 1912, but no larvae could be detected at any time up to September, 1912. Another blue tit's nest, received on the 9th October, 1911, and kept in the beehive, gave a similar record—the last living flea being found on the 7th March, 1912, but no larvae could be discovered.

A further experiment was made with *P. irritans* starting with freshly emerged adults. A large glass jar was prepared with a quantity of carpet sweepings and greasy unwashed sheep's wool and placed in the cellar on 22nd May, 1911. 20 freshly emerged *P. irritans* were fed for one week, then kept in a box in the cellar for a further week, to see if any ova would be laid immediately after the feeding. No ova were laid and the fleas were then put into the jar mentioned above.

Examinations were made periodically from September, 1911, to May, 1912, but no signs of either larvae or fleas could be found.

The cellar was chosen for the above experiments as, in consequence of the long survival period of living fleas kept there, it was thought to afford the most favourable situation for continued breeding. In order to test the possibility of egg laying under conditions exactly similar to those used to obtain eggs from fed captives, the following experiment was performed with unfed adult fleas.

A number of freshly emerged males and females of *P. irritans*, *X. cheopis* and *C. fasciatus* were placed in boxes having the cloth ring (as described in the general chapter on Methods, p. 464) and put away as follows:

Warm Cupboard. The boxes containing the fleas were kept in a moist chamber (temperature about 64° F. and the air kept as near saturation as possible).

Date	No.	Species	Remarks
15 Aug. '12	30	<i>P. irritans</i>	by the 31 Aug. all had died, no eggs were laid
"	30	<i>X. cheopis</i>	" " " "
"	30	<i>C. fasciatus</i>	" " " "

Incubator 75 Wet. Boxes containing the fleas were buried in a mass of carpet sweepings (fluff and dust) (temperature about 74° F., humidity about .81).

15 Aug. '12	15	<i>P. irritans</i>	by the 30 Aug. all had died, no eggs were laid
"	30	<i>X. cheopis</i>	" " " "
"	30	<i>C. fasciatus</i>	" " " "

Incubator 75 Wet. Boxes containing the fleas were buried in sand (temperature about 75° F., humidity .75 to .80).

18 Sept. '12	30	<i>C. fasciatus</i>	by the 5 Oct. '12 all were dead, no eggs were laid
"	30	<i>X. cheopis</i>	" 28 Sept. '12 all had died, " "

In further support of this negative result it may be noted that when flea breeding cages are kept for more than two or three months without a host for the fleas to feed on, adults will still be found present but no larvae. Cages used for breeding *C. fasciatus* were found to contain imagines 10 months after the removal of the rat but no active larvae were ever discovered.

This question is discussed at greater length in the general chapter dealing with the bionomics of fleas, Section IV, pp. 477—480.

vi. *Fertility of males and survival of spermatozoa in the spermathecae of the female.* *P. irritans*.

My interest in Rüssel von Rosenhoff's (1749) statement that fleas only copulate three times—the males not being able to fertilize after the third occasion—led me to make the following observations on captive specimens of *P. irritans*.

A single male of *P. irritans* was kept with four or five females for over a month, and though eggs were removed every three or four days there was no occasion on which all the eggs were infertile.

Two females were kept in a box with a male; the male was then taken away on the 24th July, 1910, and the females transferred to a fresh box. The change to a fresh box was repeated at intervals.

Eggs laid 28th July	to	1st August—none hatched
„ 2nd August „	5th „	„ „
„ 6th „	11th „	„ „
„ 12th „	14th „	„ „

Later a male was then added to the above mentioned two females with the following results:—

26th September 1910: 6 eggs laid, but did not hatch.

1st October 1910: 12 eggs laid from which 11 larvae hatched.

A further trial was made as follows:—

6th October 1910: The same two females and a male—10 eggs laid, at least two larvae hatched.

15th „	as above—12 eggs laid, 7 larvae hatched.
22nd „	7 „ 1 larva „
25th „	5 „ none „
1st November, 1910: „	6 „ none „
8th „	4 „ 2 larvae „

Twelve eggs were laid by a captured female some five days to a week after capture—one egg hatched.

A week or 10 days later this female laid 6 more eggs, having had no intercourse with a male—none of the eggs hatched.

Seven eggs which were laid by a virgin female kept and fed in a separate box did not hatch.

The suggestion to be gleaned from these observations was that, as a single male fertilized the eggs of at least one of a number of females for more than a month, Röscl von Rosenhoff's statement, alluded to above, must be incorrect, and further that females could not continue to lay fertile eggs after separation from the male for a few days.

The following experiment, however, made in the autumn of 1911, shows that the last conclusion was incorrect, and that females may retain the power of laying fertile eggs for a considerable period after copulation.

A number of freshly emerged females and males were put into a box and fed for 12 days, when all the males were removed and the females' oviposition recorded. The latter were kept at 75° F. when not feeding.

Date	Number of ova laid	Number hatched
19th October 1911	18	Nil
21st "	10	2
25th "	42	19
28th "	38	7
2nd November 1911	44	15
6th "	40	1
10th "	8	Nil
14th "	10	1
18th "	16	Nil
24th "	11	Nil
28th "	8	Nil
2nd December 1911	9	3
9th "	7	1
14th "	11	Nil
19th "	10	Nil

The following two series of experiments were then put in hand with a view to settling the questions raised by the earlier trials, the object being to test the fertilizing power of a male and to determine the length of time during which the female retains the power of laying fertile eggs without access to a second male.

The method employed was the following: a single male was selected out of a number of freshly emerged fleas and allowed to remain at least four days in succession with each of a number of females freshly emerged from cocoons that had been kept in separate tubes to avoid any possibility of random pairing.

The ova were removed from the boxes every three or four days. All the specimens when not feeding were kept in Incubator 75 Wet.

Fertility experiment with P. irritans. SERIES I.

The male used emerged from cocoon on 3 Feb. 1912.

Female No.	1	Date of emergence from cocoon	Male added	Male removed	Started to lay	No. of eggs laid	Number hatched	Remarks
	2	3 Feb. '12	12 Feb. '12	16 Feb. '12	4 Mar. '12	2 2	0 0	
"	3	3 "	12 "	16 "	26 Feb. '12	1 1 1	0 0 0	
"	3	3 "	16 "	20 "	24 "	2 2	0 0	
"	4	3 "	20 "	24 "	—	—	—	did not lay
"	5	3 "	24 "	28 "	8 Mar. '12	2 7	0 0	
"	6	3 "	28 "	3 Mar. '12	5 "	6 3 1	0 0 0	
"	7	16 "	3 Mar. '12	7 "	8 "	4 5	0 0	
"	8	19 "	7 "	11 "	16 "	3	0	
"	9	20 "	11 "	15 "	16 "	6	3	
"	10	29 "	15 "	19 "	—	—	—	did not lay

SERIES NO. I. In the experiments of the first series (see Table above), none of the eggs laid by the first eight females hatched. It was therefore decided to abandon the experiment, on the ground that

the male was in some way defective. This decision had already been acted on and the females added to the general stock before the ova laid by female No. 9 had hatched.

SERIES NO. II. The second series was successfully carried through. The results are of considerable interest, and show the large amount of variation that exists both in regard to the egg laying capacity of the females of *P. irritans* and the fertilizing powers of the males. While the conditions of the experiment were not exactly natural they were not unfavourable, and are likely to differ from what obtains under free conditions only as regards the restriction of the opportunities for copulation. As a measure of variation between one specimen and another the results of the experiments seem perfectly valid.

The result to be gleaned from Series II (*a*), April to June 1912 (see Table on pp. 639, 640), is that a single male is able to fertilize as many as 13 females, but that the supply of sperms passed to the female becomes exhausted within a period of from one to two months. In order to make sure that the failure of the eggs to hatch was due to the absence of a male and not to some defect in the females, a second male was placed with females Nos. 1, 2, 3, 9, 13 and 14 after they had each laid at least six batches of infertile eggs, that is after a period of about 25 to 30 days had elapsed since any fertile ova had been laid.

The sequel Series II (*b*) to Series II (*a*) lasted from June to December 1912, and the results entirely confirm those previously obtained. The course of the experiment was interrupted by a change in the food from the middle of July to the end of August 1912, when the insects were fed by my assistant during my absence from home. It will be noted that egg laying fell off at about this period, possibly owing to the change of diet, but more probably either because they did not get such full meals, or because there was a considerable fall in mean temperature during the latter half of July and throughout August, 1912. Upon my return I decided to keep the insects in incubator 75 Wet in order to finish the fertility observations, and I also instituted a separate experiment to test the effect of differential feeding.

Towards the close of the experiment the daily periods allowed for feeding were considerably increased and the eggs were allowed to accumulate for periods of 5, 6 or even 7 days before removal; it is to these causes that the apparent spurt in egg laying (see for example, females Nos. 13 and 14 in Series II (*b*)) is to be, at any rate in part, attributed, but the number of collapsed and small sized eggs suggested also some failure of control in oviposition. This may have been due

either to old age or perhaps to the unnatural condition of long continued warmth and high feeding in place of the normal state of rest or partial hibernation that occurs with this species during the winter.

The introduction of a third male in the case of females Nos. 1, 9, 13 and 14 after the lapse of the period of fertility due to the second copulation, resulted again in the production of fertile eggs by females Nos. 1, 13 and 14. It seems reasonable to suppose that the failure in the case of No. 9 was due to some defect in coitus rather than to failing powers on the part of the female to produce ova capable of fertilization. That the fault lay with the female rather than with the male seems definite as the later introduction of a fourth male did not alter the state of affairs.

It will be seen that female No. 13 was by far the most productive of those tested; she laid 448 eggs, of which 115 were fertile, in a total period of 196 days. This specimen is also to be credited with the ability to retain the power of fertilizing her eggs for the longest period, 65 days.

Fertility experiment with *P. irritans*. SERIES II (a).

The first male used emerged 1 April 1912.

No.	Female	Date of emergence from cocoon	Male added 1912	Male removed 1912	Started to lay 1912	Number of eggs laid, 1 day totals	Number of eggs which hatched, 1 day totals	Effective period of fertilization 24 days	Total number of eggs 87 in 62 days	Number of eggs fertilized 26	Percentage of fertile ova laid, calculated for period of fertility only 53 1/2	Remarks
No. 1		1 April	5 April	9 April	12 April	6 9 11 8 6 4 3 3 5	4 6 4 5 5 2 0 0 0 0	0 0 0 0				
" 2		3 "	9 "	13 "	13 "	6 6 4 6 8 7 8 8 6 2	3 5 2 3 5 4 4 3 3 2	40 "	73 "	34	55	
" 3		9 "	13 "	17 "	18 "	2 2 0 0 4 4	0 0 0 0 0 0	32 "	61 "	69	26	Copulation observed
" 4*		16 "	17 "	21 "	23 "	1 4 7 3 5 9 6 3 3 4	0 3 2 1 0 1 1 2 0 0					
" 5*		16 "	21 "	25 "	30 "	0 4 6 3 3	0 0 0 0 0					
" 6*		16 "	25 "	29 "	28 "	5 7 10 6 10 6 5	3 6 5 3 4 4 1	—	49 "	28	53	
" 7*		22 "	29 "	3 May	1 May	3 3 3 2 3	1 1 2 0 2	—	14 "	20	6	42
" 8*		25 "	3 May	7 "	5 "	7 11 10 11 14	4 3 7 5 4	—	53 "	20	23	42
" 9		25 "	7 "	11 "	11 "	4 7 7 7 3	1 6 4 4 2	—	28 "	20	17	60
" 10†		2 May	11 "	15 "	16 "	6 7 6 6	3 3 3 3	—	25 "	16	12	48
" 11†		14 "	15 "	19 "	22 "	8 8 11 7 5 6 4 7 5 4	4 4 10 5 4 2 3 0 3 3	44 days	86 "	81	39	57
" 12		19 "	24 "	28 "	8 June	3 0 3 0 6 3 6 0 0 0	1 0 0 0 0 0 0 0 0					
" 13		28 "	28 "	1 June	6 "	3 3 3 4 4 6	3 1 1 3 3 2 3	—	27 "	28	16	58
" 14		1 June	1 June	6 "	10 "	4 7 7 7 3	0 2 5 3 2	—	28 "	20	12	42
" 15		6 "	6 "	12 "	12 "	4 4 8 5 6 4 5 6	0 0 0 0 0 0 0	—	42 "	32	none	Flea removed from experiment
" 16		28 "	28 "	1 June	6 "	11 9 6 7 14 8 10 5 5	5 5 4 6 6 4 5 3 1	36 days	93 "	86	39	51
" 17		1 June	1 June	6 "	10 "	10 5 3 6 1 0 2 0 0	0 0 0 0 0 0 0 0					
" 18		1 June	1 June	6 "	10 "	9 5 4 11 7 0 5 1 6 6	0 3 2 7 3 0 2 0 1 0	36 "	61 "	93	18	37
" 19		6 "	6 "	12 "	12 "	0 4 0 0 3 0 0	0 0 0 0 0 0 0					
" 20		6 "	6 "	12 "	12 "	2 0 3 7 0 5	0 0 0 0 0 0 0	—	17 "	24	none	Male died 12 June '12 while in box with No. 15. Flea removed from experiment

* Numbers 4 to 8 removed from the experiment on 20 May '12 in order to make room for fresh females.

+ Numbers 10 and 11 also removed.

NOTE. Owing to the time involved and the difficulties in feeding when so large a number of separate boxes were required it was found necessary to limit the experiment. Nos. 4, 5, 6, 8, 10, and 11 were therefore removed on the 20th May (as events happened rather unnecessarily, as the male brought the series to a close by dying on the 12th June).

Fertility experiment with *P. irritans*. SERIES II (b).

Second male, emerged 13 June '12, died after having been placed with females Nos. 1, 2 and 3. Another male, emerged 1 Aug. '12, was used for Nos. 9, 13 and 14.

No.	Femurle	Male bred 1912	Male removed 1912	Number of eggs laid, 4 day totals	Number of eggs which hatched, 4 day totals	Effective period of fertilization 40 days	Total number of eggs	Number of eggs fertilized	Percentage of fertilite ova laid calculated for period of fertility only	Remarks
No. 1	7 10 12 9 10 7 7 15 5 3 0 0	7 10 12 9 10 7 7 15 5 3 0 0	1 3 5 5 8 1 7 11 0 1 0 0	0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0	40 days	99 in 88 days	42	49 9/10	—
" 2	0 0 0 0 0 *2 4 4 4	0 1 3	0 0 0	0 0 0	0 0 0	Nil	4 " 25 "	Nil	—	Died 17 July '12
" 3	11 9 10 8 10 3 5 4 1	11 9 10 8 10 3 5 4 1	4 4 0 0 0 0 0 0	4 4 0 0 0 0 0 0	16 days	61 " 57 "	12	40	—	" 22 Aug. '12
" 9	2 0 3 0 0 3 *9 0 6 3 3 2 2	2 0 3 0 0 3 *9 0 6 3 3 2 2	0 0 0 0 0 4 0 0 0 0 0 0	0 0 0 0 0 4 0 0 0 0 0 0	doubtful	34 " 56 "	4	doubtful	—	—
" 13	*8 11 12 10 8 10 7 6 4 5 0 8	*8 11 12 10 8 10 7 6 4 5 0 8	4 9 10 10 6 9 5 5 2 1 0 2	about 65 days	279 " 90 "	75	58	—	—	—
" 14	0 7 9 8 0 6 18 28 26 56 32	0 7 9 8 0 6 18 28 26 56 32	0 1 2 0 0 0 0 0 0 0	—	—	—	—	—	—	—
" 14	*3 12 7 7 9 2 4 2 2 0 3 0 0	*3 12 7 7 9 2 4 2 2 0 3 0 0	2 3 2 2 3 2 0 0 1 0 1 0	—	—	—	—	—	—	—
" 14	0 2 0 5	0 2 0 5	0 0 0 0	—	—	—	—	—	—	—
Third Male, freshly emerged.										
" 1	7 10 5 10 7 10 4 4 3	7 10 5 10 7 10 4 4 3	6 6 3 8 4 7 2 0 0	" 30 "	60 " 36 "	36	60	—	—	Died 28 Oct. '12
" 9	2 2 0 1 5 0 4 5 0 5 11 17 13	2 2 0 1 5 0 4 5 0 5 11 17 13	0 0 0 0 0 0 0 0 0 0 0 0	Nil	82 " 70 "	Nil	—	—	—	" 2 Feb. '13. A fourth male tried but no eggs were laid
" 13	27 10 27 12	27 10 27 12	0 0 1 0	doubtful	76 " 20 "	1	1	—	—	Died 2 Feb. '13
" 14	19 30 7 14	19 30 7 14	1 5 0 0	8 or 10 days	70 " 16 "	6	8	—	—	" 27 Dec. '12

* This mark is placed against a batch of eggs to signify that from this time onwards the females were kept in an incubator at 70° F. It was necessary to pursue this course as the cold weather had caused a cessation of egg laying.

vii. *Influence of food supply upon the number and fertility of eggs.*
P. irritans.

The following experiment was specially arranged to investigate the influence of the amount of food provided for the adult upon the number and fertility of the eggs laid. The results do not exhibit any clear connection between the opportunities afforded for feeding and the percentage of eggs hatching, but, on the other hand, show very definitely the extent to which the total number of eggs laid is dependent on food supply.

Experiment with P. irritans. The influence of food supply upon
(a) the number of eggs laid and (b) their fertility.

Method. A number of freshly emerged fleas were fed for a few days. 5 males and 5 females were selected and then put into each of two boxes. The specimens in Box No. 1 were fed once a day, those in Box No. 2 twice a day.

Box No. 1			Box No. 2		
No. of eggs laid, 3/4 day totals	No. of eggs hatch- ing, 3/4 day totals	% hatching	No. of eggs laid, 3/4 day totals	No. of eggs hatch- ing, 3/4 day totals	% hatching
23 36 49 16 44	12 25 30 11 38		23 62 98 39 55	11 32 66 31 36	
Total 168	Total 116	69%	Total 277	Total 176	63%

A female in Box No. 2 died and one was taken out of Box No. 1 for the sake of uniformity.

The feeding was reversed after the 5th batch of eggs had been removed. The individuals in Box No. 1 were fed twice a day and those in Box No. 2 once per day.

50 62 44 76 57	34 45 34 54 42		50 44 30 47 24	31 27 26 41 21	
Total 289	Total 209	71%	Total 195	Total 146	75%

SECTION VI. INFLUENCE OF LOW TEMPERATURE UPON THE VARIOUS STAGES.

Ice Chest Experiments (Table LX). *X. cheopis* bred too slowly in the cages during the cool months of the year to afford a plentiful supply of the insect in all its stages. In consequence experiments with this species were practically restricted to the summer and autumn months and, in order to investigate in detail the influence of low temperature upon the various stages in its life history, recourse was had to a small ice chest kept in the cellar. It was thought well to try *P. irritans* and *C. fuscatus* at the same time for purposes of comparison. The humidity was high—bordering on saturation—and the temperature fairly constant at about 40° F., save on a few occasions when the local supply of ice failed. In this case the extreme range was

between 35° F. and 45° F., mounting for a few hours a day to 50° F. and on one occasion to 54° F.

The results afford further evidence of greater susceptibility to low temperatures on the part of *X. cheopis* than in the case of *P. irritans* and *C. fasciatus* that was apparent in the breeding experiments and also carried out during the autumn of 1911 (Table XXV, p. 532). In all stages, save the imaginal, low temperatures below 45° F. were fatal to *X. cheopis*. As a means of destroying brood, cold air might be used as successfully as any of the usual insecticides against this species, and no further explanation is required for its failure to establish itself in cool or cold climates.

These experiments also bring out clearly the difference between *P. irritans* and *C. fasciatus* in their ability to breed under cool conditions. The record of all three species is the more interesting if the results of this experiment are compared with those obtained previously under the moderate temperatures (47—55° F.) afforded in the cellar.

Egg stage (Table LX (a)). While *C. fasciatus* can hatch at an average temperature of 41° F., *P. irritans* cannot, but a rise of 8 or 9° F. allows a percentage to hatch (see Table VI). To induce ova of *X. cheopis* to hatch a temperature of over 60° F. is apparently necessary (see Table VII, p. 497).

Larval stage (Table LX (b)). Here again it will be noted that, while *X. cheopis* and *P. irritans* cannot survive below 40° F., *C. fasciatus* is not only able to endure but apparently finds this temperature quite suited to its needs. The only factor that prevented all the larvae being reared to cocoon stage was the length of time taken by lagging larvae.

Cocoon stage. At 40—43° F. the results were similar to those obtained with the larvae. The greater endurance of *P. irritans* than *X. cheopis* appears from the fact that five of the former reached the pupal or imaginal stage before death, but only one of the latter survived the larval period.

Adult stage. The disparity here is not nearly so marked, *X. cheopis* being nearly or quite as hardy as *P. irritans*, while all three species are more nearly alike in this stage than in any previous one.

TABLE LX. Ice chest experiment. Relative endurance at low temperature of *X. cheopis*, *P. irritans* and *C. fasciatus* at various stages in their life history.

General record of Temperature and Humidity during the experiment.

General record of Temperature and Humidity		Temperature		Average	Humidity				
		Highest	Lowest						
Date	Month	(Average of 31 readings)							
7 June '12	April '12	49	35	(" 53 ")	39.3	.92			
"	May	54	35	(" 50 ")	39.3	.95			
"	June	50	35	(" 43 ")	40.9	.99			
"	July	60	37	(" 43 ")	43.6	.95			
(a) Eggs, laid in Incubator 75 Wet.									
Date	Species	Number	Temperature		Humidity	Number hatched	Mortality 100%		
			Extremes	Aver.					
7 June '12	<i>X. cheopis</i>	10	50° 35°	40.9	.99	Nil	100		
"	<i>P. irritans</i>	10	50° 35°	40.9	.99	Nil	100		
"	<i>C. fasciatus</i>	10	50° 35°	40.9	.99	5	50		
(b) Larvae, newly hatched in Incubator 75 Wet, unfed, placed in small glass tubes containing a little sand and plugged with cotton wool.									
Date	Species	Number	Temperature		Humidity	Length of life, days		Average length of life	
			Extremes	Aver.		Minimum	Maximum		
29 April '12	<i>X. cheopis</i>	6	40° 36°	38.2	.97	2	3	2 days	
"	<i>P. irritans</i>	9	49° 35°	37.0	.95	2	46	13 "	
"	<i>C. fasciatus</i>	8	54° 35°	39.0	.96	9	52	43 "	
Active (feeding) larvae of various sizes taken from the cages (or in the case of <i>P. irritans</i> from stock pot in Incubator 75 Wet) and placed in card jars with sand and blood-soaked rag.									
Date	Species	Number	Temperature		Humidity	Number of cocoons found	Number of fleas reared	Interval during which larva were observed	Mortality 100%
			Extremes	Aver.					
15 April '12	<i>X. cheopis</i>	32	46° 37°	40	.94	none	none	No larvae seen alive after 3 days	100
"	<i>P. irritans</i>	22	49° 37°	41	.93	"	"	" " 8 "	100
"	<i>C. fasciatus</i>	22	49° 35°	April 39.3	.92	16*	—	" " 56 "	No mortality during the course of experiment
			54° 35°	May 39.3	.95				
			50° 35°	June 40.9	.99				
			60° 37°	July 43.6	.95				

* 16 Aug, 1 flea emerged; 6 Sept., 2 cocoons opened contained resting larvae; 9 Sept., 13 cocoons opened contained resting larvae.

TABLE LX.—Continued.

(c) Cocoons, spun by larvae in Incubator 75 Wet.									
Date	Species	Number	Temperature Extremes	Aver. Temp.	Humidity	Number of fleas which emerged	Aver. number of days in cocoon	Remainder of cocoons opened and found to contain	
15 April '12	<i>X. cheopis</i>	23	April 49° 35°	39·3	·92	mortality at least 96 1/10	—	Two larvae came out of cocoons and died within the first few days. Remaining cocoons opened 26 July contained 20 dried up larvae and 1 living pupa (transferred to In- cubator 75 Wet, since died)	
"	<i>P. irritans</i>	18	May 54° 35°	39·3	·95			Three larvae came out of their cocoons, two died, one pu- pated. Remaining cocoons opened 26 July contained 2 dried up fleas, 2 dried up pupae, 11 dried up larvae	
"	<i>C. fasciatus</i>	22	June 50° 35°	40·9	·99	mortality 100 1/10	—	Remaining cocoons transferred to cellar 26 July	
"			July 60° 37°	43·6	·95				
Remaining cocoons opened 9 Sept. contained 5 resting larvae, 2 empty ("false" cocoons).									

(d) Adult fleas, newly emerged and unfed, kept in card jars with sand at bottom to burrow in.

Date	Species	Number	Temperature Extremes	Aver. Temp.	Humidity	Length of life, days Minimum	Maximum	Average length of life, days
29 April '12	<i>X. cheopis</i>	11	54° 35°	39·3	·95	7	36	20
"	<i>P. irritans</i>	4	54° 35°	39·3	·95	7	38	16
"	<i>C. fasciatus</i>	11	54° 35°	39·3	·96	7	51	34

SUMMARY.

Eggs and egg-laying.

In comparison with the later stages in life history eggs are relatively insusceptible to external conditions. The range of variability in the length of time between laying and hatching, 2 to 10 days, is a small one and there is no appreciable tendency to utilize this stage for resting, the response to temperature changes being simple and direct.

The upper limit of temperature which is fatal to eggs has not been determined. *C. fasciatus* showed a high percentage hatching at 85° F. (see Table IV), while in the case of *P. irritans* 9% hatched at 93° F. (see Table VI a), and on one occasion (Table VIII, series III) 27% of the eggs of *X. cheopis* hatched at 93° F. At low temperatures the numbers which hatch in the case of all three species is reduced and cold is fatal to eggs of *P. irritans* and *X. cheopis*. In the ice chest, at an average temperature of 40·9° F. (see Table LX), 50% of the eggs of *C. fasciatus* hatched, while all those of *X. cheopis* and *P. irritans* failed. A small proportion of eggs of the latter species hatched at 46° F., but in the neighbourhood of 55° F. appears to be the minimum for *X. cheopis*.

As regards the influence of humidity upon the hatching of eggs, in the range used in these experiments, only the larger differences produced consistent results, and, although there were some complete failures of hatching under conditions of low humidity, the evidence does not seem clear enough to warrant these being considered conclusive tests. On the whole, it is safe to say that a temperature of 65° to 80° F. with a humidity of ·70 or over is most favourable and that if the temperature be above 60° F. humidities below ·50 to ·55 are harmful. In the case of *P. irritans* there is possibility of complete failure at a humidity of ·50 but 70% of the eggs of *C. fasciatus* hatched at a temperature of 75° F. with a humidity of only ·48 (see Tables II and IV).

Females of *C. fasciatus* show, in some cases, a fall in egg production under conditions of drought, but the effect is not noticeable with *P. irritans* and *X. cheopis*. Low temperatures check or prevent oviposition.

In the case of *C. fasciatus* and *P. irritans* warmth (75° F.) combined with low humidity, favours the fertility of eggs laid. This, in the case of *C. fasciatus*, might possibly be explained on the assumption that the fertility of ova is likely to be in inverse ratio to the number laid (see Table IV), but, in the case of *P. irritans* (Table VI) no such explanation is possible and we are forced to the conclusion that drought

is in some way, perhaps by acting on the instinct to copulate, responsible for an increase in the percentage of fertilized eggs. With *X. cheopis* drought does not seem to have any marked effect on the fertility of eggs (see Table VIII).

Larvae.

This stage may be subdivided into two periods, (1) an active and (2) a quiescent or resting phase, passed within the cocoon.

In the present work the second or resting period has, for convenience, been treated under a joint heading with the cocoon stage. For such treatment, there is some justification, for no genuine metamorphic stages could be more sharply separated from one another as regards power of resisting unfavourable conditions and death than are the periods of active and resting larval life respectively. The range of conditions which the active larvae are fitted to surmount is a narrow one, but, once within the cocoon, the species studied can easily tide over dangers that would mean 100 % mortality in the active stage.

Reference to Table XXV will show that in the dry incubators (75° F. and 84° F. and humidity '60) and warm cupboard (67°—69° F. and humidity '65—'71) active larvae of *P. irritans*, as well as of *C. fasciatus* and *X. cheopis*, died, while in the cocoon stage some survived. The fact that in these dry situations the majority of the insects which died in the cocoons were in the larval stage suggests that the transference of cocoons took place without allowing a sufficient interval of time to elapse after spinning.

In the case of all the species investigated, the newly hatched larvae were able to live from several days to over a month without food, provided the conditions were not otherwise unfavourable. Given food and reasonable conditions of temperature, the next important requirement is as high a percentage of moisture in the air as is compatible with surroundings so dry that there is no danger of a wet skin. Local moistening by the urination of animals or sweat from their bodies may convert what would otherwise be an impossible place into a favourable situation; while draughty conditions, with a comparatively high humidity, may be less favourable than a drier situation with a nearly still atmosphere¹.

Evidence has been found of a difference in the kind of food necessary for *C. fasciatus* on the one hand and *P. irritans* and *X. cheopis* on the other. That the faeces of the adult fleas are a possible diet and

¹ This point may be of importance, as well ventilated houses will be far less suitable for flea-breeding than ill ventilated ones.

favourable for the larvae of all three species has been proved. At the same time there is some evidence which points to this food being a necessity for *C. fasciatus*. This divergence in the matter of food among larvae of the different species is consistent with the habits of the parents and the parental host.

The active larval period of *C. fasciatus* is not necessarily one of steady growth or continuous feeding, as larvae have been found from several days to two or three weeks after hatching without any appreciable increase in size. Even when full growth is attained a period of apparently unnecessary delay, or "lagging," sometimes occurs before the spinning of the cocoon is commenced (see Table XXVI). This mode of lengthening the larval stage probably occurs also with *P. irritans*, and possibly with *X. cheopis* as well.

The time occupied by the active larval period is subject to very wide variation, ranging from 15 to 114 days for *C. fasciatus* (Table XVI), 9 to 202 days for *P. irritans* (Table XXI), 12 to 84 days for *X. cheopis* (Table X), and 11 to 142 for *Ct. canis* (Table XXIV). Although low temperature is responsible for the instances of very protracted active life, the question of temperature is not the sole factor determining the length of the period elapsing between the hatching of the egg and the spinning of the cocoon, for marked individual variations occur in this respect between larvae from the same batch of eggs, reared under identical conditions.

Cocoons.

The duration of the cocoon period varied from 8 days to well over a year for *C. fasciatus* (Table XXVIII), from 7 to 239 days for *P. irritans* (Table XXXVIII), from 7 to 182 days for *X. cheopis* (Table XXXV), and from 7 to 354 days for *Ct. canis* (Table XL).

The question of how much of this period is spent as a larva, how much as a pupa and how much as a flea awaiting some stimulus to make it emerge, is no easy matter to determine. It is certain that *C. fasciatus* will rest for long periods as a larva—I have records up to 600 days. With the other species, however, the matter is far from being definitely settled. The observations indicate that the resting stage of *Ct. canis* is generally, if not always, imaginal. In the cases of *P. irritans* and *X. cheopis* the evidence is less definite but points to a rest in the imaginal state before emergence, when this is long delayed. I am of opinion, however, that a considerable period of the time elapsing between the spinning of cocoons and emergence is passed in the larval

condition. I have no evidence that the pupal period is ever utilized for resting. So far as can be ascertained, the development in the pupa, though delayed by cold and hastened by heat, is continuous until the pupal envelope is shed.

The length of the cocoon period is largely determined by conditions of temperature, but the range of variation, under exactly similar conditions, was found to be very considerable.

There is some evidence that a fall in temperature during larval life predisposes both *X. cheopis* (see Tables XXXIV and XXXV) and *P. irritans* (see pp. 537, 538) to lengthen their cocoon period. The results of the experiments with *C. fasciatus* (Table XXXII (b)) do not suggest that this happens in the case of this species. Table XXXIII, however, shows a predisposition on the part of November batches of larvae of this species to rest longer in the cocoon stage, apart from the actual condition of temperature obtaining at the time the cocoons were spun.

The cocoons of different species differ both in shape and texture, but the outward appearance is largely dependent upon the material in which the larvae live, fragments of which become attached during the spinning. The cocoons of *C. fasciatus* vary greatly as regards the texture and quality of the silk used, some being frail and loose, others as hard and brittle as though composed of glue rather than silk. There is good reason to conclude that hard cocoons are associated with lengthy resting periods (see pp. 610—612).

Ct. canis shows a strong disposition to spend the winter in the cocoon stage. In the case of *P. irritans* there is the same tendency, but it is less obvious, individuals continuing to emerge throughout the winter, if the weather is mild. *C. fasciatus* also frequently passes the cold weather in the cocoon, and a certain proportion of individuals of this species are in the habit of aestivating during the hot months and emerging during the cooler weather of autumn. In hot climates this habit might easily afford a basis for selective action and the consequent production of a race composed of individuals, displaying on the one hand a quick, and on the other greatly delayed, emergence without intermediate types. That this is a strongly ingrained habit is suggested by the emergence of individuals after periods of 100 to 150 days in the cocoon, at a temperature of from 75° F. to 85° F. Even at 93° to 95° F. a certain number of *C. fasciatus* are able to survive as resting larvae for periods of four months and then successfully complete their metamorphosis if removed to a lower temperature (p. 606).

Although with all the species the periods of feeding as larvae and of resting in cocoons are prolonged as the temperature falls, the optimum varies in the different species, being 5° higher for *P. irritans* and 15° to 20° higher for *X. cheopis* than for *C. fuscatus*.

Individual variation, as regards the time taken by these larval and cocoon stages, ensures the spreading of the adult emergence of rat and human fleas over a very wide interval of time. In the case of *Ct. canis* there would seem to be much more restriction for any one brood, although, with this species, some individuals of a brood emerging in autumn will, under suitable conditions, lie over the winter in their cocoons. It is evident that the protection afforded by the cocoon would be the means of saving this species from extermination, not only under conditions of drought (Table XLIV), but also under circumstances of excessive moisture, for it was proved that, in this stage, actual inundation could be survived for at least 12 hours.

Adults.

At 45°—50° F. with nearly saturated air, fleas can live for many days unfed; specimens of *P. irritans* have survived for 125 days, *C. fuscatus* for 95 days, *X. cheopis* for 38 days, *Ct. canis* for 58 and *C. gallinae* for 127 days (see Tables XLV, XLVIII and L). Yet under what might for insects be considered only moderately unfavourable conditions of temperature and humidity their powers of endurance are but slight in the absence of food. *X. cheopis* is little, if any, better fitted than *C. fuscatus* to withstand heat and drought in the absence of a host, but its habit of living on the rat rather than in its bed enables this species to extend the period of its active existence both in time and space and renders it a far more dangerous agent in the spread of plague.

Although kept in a box, if fed on their natural host, *P. irritans* may live for upwards of 513 days (Table LIV), *C. fuscatus* for 106 (Table LV) and *X. cheopis*, fed on man, for 100 (Table LVI). It is probable that both rat fleas, under natural circumstances, would live longer. *Ct. canis* and *C. gallinae* have lived for periods of 234 and 345 days respectively (Table LVII) when fed on man, and it is unlikely that they have a less lengthy life when fed on dogs or fowls.

In view of the suggestion that adult fleas imbibe fluids other than blood, some experimental tests were undertaken. Dissections were made, after an opportunity had been given the fleas to imbibe

coloured fluids, but no sign of the insects having fed could be traced. Among the observations on record, which suggest that adult fleas can be nourished otherwise than by warm-blooded animals, are those in which sick flies and Lepidopterous larvae are said to have been attacked¹. I have made experiments with *C. fasciatus* and *Tenia fenestrella*. The results were negative and the fleas died as soon as the controls. The opinion I have formed as a result of my own experience is that feeding on warm blooded animals is essential to reproduction.

Most, if not all, the species dealt with have been observed to copulate shortly after emergence, but no signs of either eggs or brood have been observed in any of the boxes, jars, or other receptacles in which unfed fleas have been kept. Experiments conducted in specially prepared jars, containing food for any larvae that might result, and also abundant cover for the fleas, gave the same result. On the other hand, virgin females of *P. irritans*, when fed, laid freely, though only infertile eggs. In fact, during the course of these experiments, no support whatever has been obtained for the theory that flea breeding can take place from one generation to another in the absence of an animal host to provide food for the adult.

Although the several species of fleas other than human were successfully fed on man and lived for considerable periods, no eggs were laid by any of the species, save *C. gallinae*, when 15 minutes' daily feeding was permitted. Later attempts with the hair of the correct host placed in the boxes to afford cover, were also ineffective, but feeding for longer than 15 minutes daily, was sufficient to keep *P. irritans*

¹ The feeding by adult fleas on the larvae of Lepidoptera, noted by Boden (1882), seems to be just within the bounds of possibility, but is probably of rare occurrence. Most, if not all the Lepidopterous larvae, likely to be found in the same habitat as fleas, live in silken galleries, a habit which would afford great protection as the larvae are able to travel rapidly away from any spot where the silken tube may be pierced. The possibility of starving adult fleas attacking larvae of their own species has been specially considered as such a habit might lead to infection of the adult by any bacteria that might be living in the larval gut, and thus have an important bearing on the recrudescence of plague. I made the following trial: A number of unfed adult *C. fasciatus* were divided into two batches and placed in similar tubes and into one only of the tubes a number of flea larvae were placed. The length of life of the fleas in the tube containing the larvae was no longer than that of those in the control, while the larvae remained healthy and spun their cocoons as usual. The constant attention demanded by other work prevented any very close watch being kept on the activities of the fleas and I cannot state if any attempts were made to feed on the larvae.

Russell (1913) makes mention of attacks made by fleas on flies incapable of flight. It seems, however, improbable that this or any other similar food can be a serious factor in tiding the species over periods when no warm blooded hosts are available.

laying freely and fertile eggs were also obtained from *C. fuscatus* and *X. cheopis*. *Ct. canis* required to be given opportunity to feed for from 5 to 12 hours' daily to induce egg-laying. While *X. cheopis* feeds on man more readily than *C. fuscatus*, the females do not lay so freely, presumably because, in accordance with this species' practice of living upon its host, rather than in its nest, they require more frequent feeding than *C. fuscatus*.

The experiments in which the opportunities offered to adults of *P. irritans* to feed were varied (see p. 641) afforded no evidence that the amount of food taken had any influence on the fertility of the eggs laid, but only upon the number. This result confirms those obtained when rat and dog fleas were fed on a human host (see p. 479), and shows the great influence upon egg-production of the quantity of food taken by the adult. At the same time it further emphasises the improbability of breeding by unfed fleas.

It has been proved conclusively (p. 637) that male fleas are able to impregnate at least 13 females, and further that the females must pair more than once if the large number of eggs they are able to produce are to be of any service to the species.

It is uncertain whether the individuals that rapidly attain the imaginal stage are long-lived as adults, but it has been proved with regard to *P. irritans* that a long cocoon stage may be followed by a very long life as an adult.

My observations on the maximum duration of the various stages in the life history of *C. fuscatus*, *X. cheopis*, *P. irritans*, *Ct. canis* and *C. gallinae* may be summed up as follows:

C. fuscatus—egg stage 10 days, larval 114, cocoon 450, adult when fed 106, unfed 95.

X. cheopis—egg stage 10 days, larval 84, cocoon 182, adult when fed 100, unfed 38.

P. irritans—egg stage 12 days, larval 202, cocoon 239, adult when fed 513, unfed 125.

Ct. canis—egg stage 8 days, larval 142, cocoon 354, adult when fed 234, unfed 58.

C. gallinae—egg stage 7, larval (estimate) 50, cocoon (interrupted by opening) 70, adult when fed 354, unfed 127.

Adding together the maximum periods recorded for each stage, a fair indication of the possible length of life of the individual from egg until its death as a perfect insect is: for *C. fuscatus* 680 days, *X. cheopis* 376, *P. irritans* 966, *Ct. canis* 738, *C. gallinae* 481.

On this basis, and allowing for the longest recorded *unfed* imaginal lives, it will be seen that there is no difficulty in accounting for active adults being found, in favourable situations, where there have been *no hosts for considerable periods*. We may safely estimate for *C. fasciatus* 22 months, *P. irritans* 19 months, *X. cheopis* 10 months, *Ct. canis* 18 months, *C. gallinae* 12 months.

NOTE.

It is a matter of regret to me that the valuable and interesting paper "Observations on Flea breeding (*X. cheopis*) in Poona" (Report LV of the Advisory Committee, Plague Supplement II, *Journal of Hygiene*, Vol. XI, pp. 300—325) was not available before I had finished the experimental work and the writing of this report.

Notwithstanding the fact that the methods and conditions applying to the two series of experiments were so diverse, the great importance of humidity in controlling egg laying and hatching, the rearing of larvae and the length of the adult life is demonstrated alike by the Poona experiments and those of the present research which were carried out at Loughton, Essex. The Loughton experiments show, in addition, that both high and low temperatures are detrimental to *X. cheopis*, cold being especially fatal to the immature stages, a fact that the equable temperature of Poona tends to conceal.

The striking divergences in detail of the two sets of experiments are as follows, and must, I think, be attributed to the causes given below:

1. *Egg laying*. The much smaller number of eggs per female laid in Loughton must be attributed to the comparatively low temperature at which the cages were kept.

2. *Egg hatching*. The comparison is between ova obtained from excited insects struggling for foothold at Poona and those of individuals more comfortable, if less vigorous owing to the lower temperature, at Loughton. Under the most favourable circumstances only 49.1% hatched at Poona while the best Loughton record is 76%.

3. The greater larval mortality and shorter adult life at Poona is probably due partly to the use of methods less favourable to the insects than those employed at Loughton, and partly to a probable greater daily fluctuation of humidity at Poona.

In conclusion, I desire to record my thanks to many friends for their assistance and advice. I wish also to record my indebtedness to my assistant Mr H. J. Turner, for his valuable help and the care with which he entered up the records.

A. W. B.

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DESCRIPTION OF PLATES XXVII—XXXIV.

Plates XXVII—XXIX, photographs of various fleas magnified 30 diameters. (The specimens are treated with hot 20 % caustic potash for a few minutes, dehydrated in alcohol, cleared in xylol and mounted in balsam.)

Plate XXVII. Fig. 1. *Ceratophyllus fasciatus*, ♂.

„ 2. „ „ „ ♀.

„ 3. *Leptopsylla musculi*, ♂.

„ 4. „ „ „ ♀.

Plate XXVIII. Fig. 1. *Pulex irritans*, ♂.

„ 2. „ „ „ ♀.

„ 3. *Xenopsylla cheopis*, ♂.

„ 4. „ „ „ ♀.

Plate XXIX. Fig. 1. *Ctenocephalus canis*, ♂.

„ 2. „ „ „ ♀.

„ 3. „ „ *felis*, ♂.

„ 4. „ „ „ ♀.

Plate XXX. Fig. 1. Ova, *Xenopsylla cheopis*, × 30.

„ 2. „ „ *Pulex irritans*, × 26.

„ 3. „ „ *Ceratophyllus fasciatus*, × 26.

„ 4. „ „ *Ctenocephalus canis*, × 30.

Plate XXXI. Fig. 1. Ova, *Pulex irritans*, × 60.

„ 2. Skin of full-grown larva of *Ceratophyllus fasciatus*, × 17, showing the arrangement of dorsal plates and hairs.

„ 3. Head of *Xenopsylla cheopis*, × 90, showing the epipharynx and mandibles the elements which together form the piercing and sucking tube and beneath these the labium. The maxillary palps are in a drooping position.

Plate XXXII. Living larvae, × 10.

Fig. 1. Dorsal, lateral and ventral aspects, *Xenopsylla cheopis*.

„ 2. Lateral aspect, *Ceratophyllus fasciatus*.

„ 3. In natural surroundings seen from above, *Xenopsylla cheopis*.

„ 4. Lateral aspect, *Pulex irritans*.

Plate XXXIII. Fig. 1. Cocoons of *Xenopsylla cheopis*, × 10.

„ 2. „ „ *Pulex irritans*, × 10.

„ 3. „ „ *Ceratophyllus fasciatus*, × 10.

„ 4. „ „ *Leptopsylla musculi*, × 10.

Plate XXXIV. Fig. 1. Pupae of *Xenopsylla cheopis*, ♂ and ♀, living, × 10.

„ 2. „ „ *Ctenocephalus canis*, ♀, living, × 16.

„ 3. „ „ *Leptopsylla musculi*, ♀, living, × 16.

„ 4. Cocoons of *Ctenocephalus canis*, × 10.

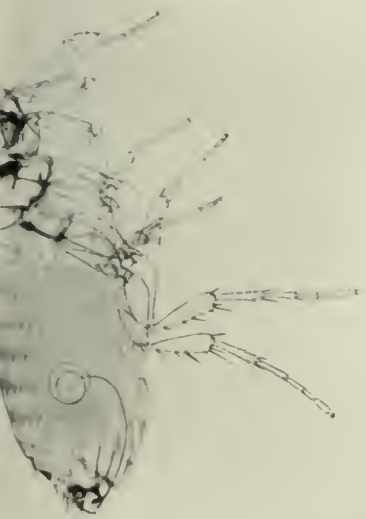


Fig. 1. *Ceratophyllus fasciatus* ♂ $\times 30$.



Fig. 2. *Ceratophyllus fasciatus* ♀ $\times 30$.



Fig. 3. *Leptopsylla musculi* ♂ $\times 30$.



Fig. 4. *Leptopsylla musculi* ♀ $\times 30$.

Photos by A. E. Tonge
Printed by A. E. Tonge



Fig. 2. *Pulex irritans* ♀ × 30.



Fig. 1. *Pulex irritans* ♂ × 30.





Fig. 2. *Ctenocephalus canis* ♀ × 30.



Fig. 1. *Ctenocephalus canis* ♂ × 30.





Fig. 1. Ova of *Xenopsylla cheopis* $\times 30$.



Fig. 2. Ova of *Pulex irritans* $\times 26$.



Fig. 3. Ova of *Ceratophyllus fuscatus* $\times 26$.



Fig. 4. Ova of *Ctenocephalides canis* $\times 30$.



Fig. 1. Ova of *Pulex irritans* $\times 60$.



Fig. 2. Skin of full grown larva of *Ceratophyllus fasciatus* $\times 17$. Showing arrangement of dorsal plates and hairs.



Fig. 3. Head of *Neopsylla cheopis* $\times 90$.

Photos by A. E. Tonge



Fig. 1. Dorsal, lateral and ventral aspects of larvae of *Xenopsylla cheopis* $\times 10$.



Fig. 2. Larvae of *Ceratophyllus fasciatus* $\times 10$.



Fig. 3. Larva of *Xenopsylla cheopis* in natural surroundings $\times 10$.
Photos by A. E. Tonge



Fig. 4. Larvae of *Pulex irritans* $\times 10$.



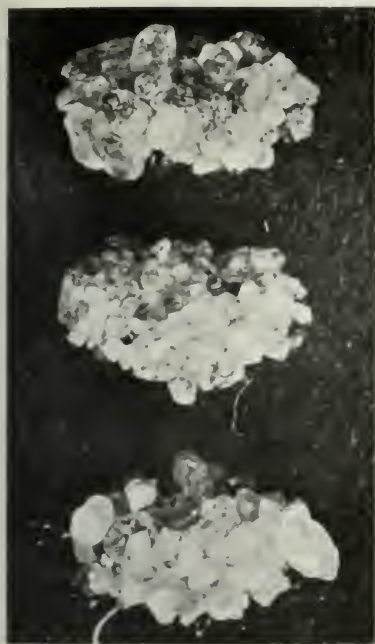


Fig. 2. Cocoons *Pulex irritans* $\times 10$.



Fig. 1. Cocoons *Xenopsylla cheopis* $\times 10$.

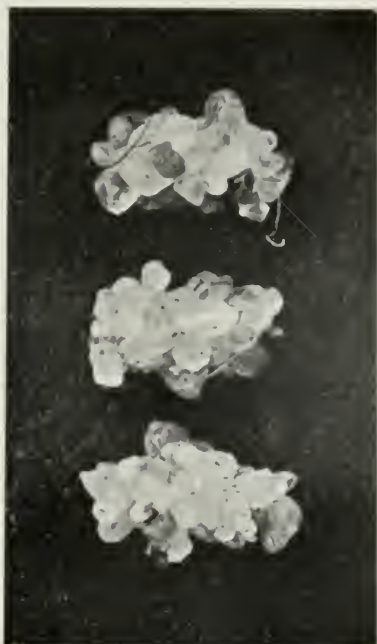


Fig. 4. Cocoons *Leptopsylla muscili* $\times 10$.



Fig. 3. Cocoons *Ceratophyllus fasciatus* $\times 10$.

Photos by A. E. Tonge





Fig. 1. ♂ and ♀ pupae of *Xenopsylla cheopis* × 10.



Fig. 2. Pupae of *Ctenocephalatus canis* × 16.



Fig. 3. Pupa of *Leptopsylla muscui* × 16.

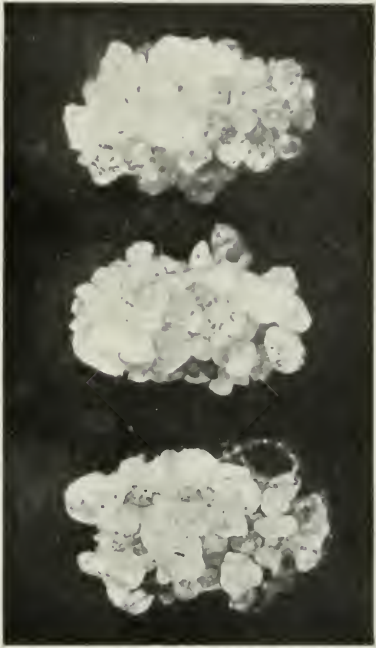


Fig. 4. Cocoons of *Ctenocephalatus canis* × 10.

Photos by A. E. Tonge



THE INFLUENCE OF THE TOTAL FUEL-VALUE OF A DIETARY UPON THE QUANTITY OF VITAMINE REQUIRED TO PREVENT BERI-BERI.

BY

W. L. BRADDON, M.B., AND E. A. COOPER, D.Sc.
F.R.C.S.

(From the Lister Institute.)

(*Preliminary Communication.*)

It is now taken as settled that beri-beri is essentially privatory in origin, resulting from the deficiency in the food supply of a substance the precise nature of which has not yet been ascertained.

If this simple explanation were complete, it would follow that the more thorough the withdrawal of the active substance, the speedier would be the onset of the disease and the more severe its effects. Several investigators, however (Maurer,⁵ 1907; Cooper,³ 1913; Caspari and Moszkowski,² 1913, and Funk,⁴ 1914), have shown that the onset of polyneuritis in birds can be hastened by increasing the ration of polished rice. From this it appears that when the food ration is increased by the addition of carbohydrate material, for example, polished rice, with a very low content of antineuritic substance, although the daily allowance of the essential substance is slightly increased, the development of polyneuritis is actually accelerated.

Analogous observations have been made in the course of the epidemiological studies of human beri-beri. Thus it has been found by nearly all observers¹ that in epidemics of beri-beri the well nourished are the first to succumb, and are actually more liable to the disease than the underfed. Similarly, under natural conditions men, owing to their larger energy output, partake of polished rice more freely than women, and are more liable to beri-beri. In institutions, however, where men and women have the same fixed ration they are equally susceptible to the disease. Again, in prisons and asylums, in which the dietary has been varied from time to time, every increase in the polished rice component of the diet, the ration of other foodstuffs remaining constant, has been followed by increased beri-beri.

From these observations Braddon drew the conclusion that in rice-eating communities the extent and severity of beri-beri vary directly with the quantity absolute or relative of polished rice consumed.

Admitting, then, that in the production of beri-beri a deficiency in the diet of a certain substance is an essential factor, it is evidently of practical importance to ascertain the part played by the substance in metabolism, and, if possible, to determine the quantitative relations which the intake of active material must bear to that of the other components of the dietary, in order that the requirements of the organism can be covered.

With this object in view a study has been made of the effect upon the onset of polyneuritis in birds of increasing the carbohydrate ration of the dietary, and the main experimental results are briefly summarized in this preliminary communication, and will be shortly published *in extenso* in the *Journal of Hygiene*. The observations were made at Seremban, Federated Malay States, during the years 1909-11, and at the Lister Institute during 1913-14.

It is now a well-established observation that chickens fed on $\frac{1}{20}$ their body-weight of polished rice develop polyneuritis in twenty to seventy days, whereas those fed on the same daily ration of unpolished rice (padi), "cured" or parboiled rice, and "fresh" rice (partly polished) remain free from the disease. Braddon found, however, that birds when fed on $\frac{1}{20}$ their body-weight of parboiled rice, which has been soaked for twenty-four hours in water and then strained off, develop polyneuritis just as when fed on ordinary polished rice, and epidemics of beri-beri have been traced to the practice of soaking unpolished rice prior to cooking, and discarding the water.

The effect of adding carbohydrate to a diet which itself was adequate to maintain birds in health was first studied, and experiments made on forty birds showed that, while chickens fed on $\frac{1}{20}$ their body-weight of padi remained free from polyneuritis for at least sixty to a hundred days, when this ration was supplemented by $\frac{1}{4}$ the body-weight of washed parboiled rice or by $\frac{1}{40}$ the body-weight of polished rice the birds contracted the disease in twenty to thirty days.

Experiments were next carried out with the object of ascertaining more precisely what effect increasing the carbohydrate component of a dietary had upon the rate of onset of polyneuritis. In these experiments altogether thirty birds (chickens and pigeons) were employed.

It was found that:

1. Whereas pigeons fed on $\frac{1}{20}$ their body-weight of polished rice and $\frac{1}{2500}$ of dry yeast daily developed polyneuritis in from thirty-nine to ninety-five days, when the ration of rice was increased to $\frac{1}{5}$ the body-weight the birds fell ill in twenty-two to forty-six days.
2. While pigeons fed on $\frac{1}{20}$ their body-weight of polished rice and $\frac{1}{3500}$ of yeast developed polyneuritis in from

thirty-two to forty-four days, they became affected with the disease in from fifteen to twenty-two days when the polished rice ration was increased to $\frac{1}{10}$ the body-weight.

3. Whereas chickens fed on $\frac{1}{40}$ their body-weight of polished rice and $\frac{1}{2500}$ of yeast did not fall ill with polyneuritis until from sixty-five to one hundred days had elapsed, when this dietary was supplemented by $\frac{1}{10}$ the body-weight of sago the birds contracted the disease in from thirteen to thirty days.

By doubling the carbohydrate ration of the dietary the rate of onset of polyneuritis was thus actually increased as much as from two- to four-fold.

The results so far obtained demonstrated that the amount of antineuritic substance required by the organism increases with the quantity of carbohydrate ingested.

There are at least two possible explanations of this phenomenon. First, the view already advanced by Funk (1914) that the active substance participates in some way in carbohydrate metabolism, and thus the more metabolic work the organism is called upon to undertake the greater its demand in respect of this essential substance. Secondly, that when large rations of starch are ingested the absorption of the antineuritic material is interfered with owing to the presence in the alimentary canal of excess of undigested carbohydrate.

Experiments were therefore next carried out to ascertain which of these was the true interpretation, and it was found that even when daily rations of polished rice as great as $\frac{1}{10}$ the body-weight were fed to chickens together with varying amounts of yeast, 93 to 98 per cent. of the starch was digested and absorbed, and the excreta were free from the antineuritic substance. It was also observed that *in vitro* starch did not absorb the substance from aqueous solution. Furthermore, pigeons fed exclusively on glucose, which is rapidly absorbed, developed polyneuritis. These observations exclude the physical factor, and show that the antineuritic substance is utilized in some way during carbohydrate metabolism.

Whatever be the mechanism involved, it is clear that for the maintenance of health the intake of active substance must be adjusted in relation to the ration of carbohydrate supplied, and it is when this necessary balance is not maintained in the dietary that beri-beri results.

The precise relation which must subsist between the supply of antineuritic substance and the amount of carbohydrate ingested has not yet been ascertained, nor has the corresponding relation for each of the other normal components of a dietary (protein and fat) been determined. Funk (1914), however, has made some experiments which indicate that protein and carbohydrate can be interchanged in a dietary without materially affecting the rate of onset of polyneuritis, whereas if these food materials be replaced by fat the disease develops less readily.

The fact that attention must be paid not merely to the absolute amount of antineuritic foodstuff incorporated in

the dietary but also to the proportion which this bears to its total calorific value is of great practical importance in framing a dietary for the prevention of beri-beri.

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³Cooper, 1913: *Journal of Hygiene*, xii, 4, 436. ⁴Funk, 1914: *Zett. physiol. Chem.*, 89, 3, 378. ⁵Maurer, 1907: *Muench. med. Wochenschr.*, liv, 371.

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XXXII. THE FACTORS CONCERNED IN THE SOLUTION AND PRECIPITATION OF EUGLOBULIN.

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From the Lister Institute.

(Received April 15th, 1913.)

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I. INTRODUCTION.

There is no reason for regarding euglobulin, the material precipitated from serum by dialysis or by dilution and acidification, as a chemical entity. Michaelis and Rona [1910, 2] are of opinion that it consists of that portion of the total proteins which owes its solution to the dispersing power of the electric charge upon its particles. It is true that the procedure adopted for separating euglobulin is one which renders it iso-electric with the solution; but the conditions determining its dispersion in serum are not so simple as they suggest. Euglobulin, as shown by Hardy [1905], is dissolved by neutral salts, e.g., sodium chloride, in a concentration of 1/10th normal, to form a colloidal solution in which the particles are electrically neutral, a result which I have been able to confirm (see p. 329, below). Serum contains enough salt to produce this effect and unless it is at the same time diluted, the euglobulin it contains cannot be completely precipitated by the addition of the amount of acid necessary to render it iso-electric. The dispersion of euglobulin in serum is due to two entirely distinct causes: (1) Electric charge on the particles owing to the alkalinity of the fluid: (2) Formation of a "soluble" compound with the salt present,

concerning the mechanism of which there are a variety of theories, which are discussed in detail below.

By the process of "heat-denaturation" proteins acquire many of the properties peculiar to euglobulin, as regards the factors conditioning their solution (dispersion) and agglutination (see Hardy [1900], Michaelis and Mostynski [1910] and Chick and Martin [1912]). The results of the last-named, however, afford no support for the opinion expressed by Starke [1900] and Moll [1904] that "albumin" is converted into "globulin" by heating. Abderhalden [1903, 1904] has found evidence of difference in chemical composition between the two sets of proteins which is not obliterated after the former has been heated. Further, the analogy mentioned above is by no means complete; the union of euglobulin with neutral salts to form a dispersion that is without drift in an electric field has no parallel in the case of denaturated serum-proteins.

II. SOLUTION OF EUGLOBULIN BY ACIDS AND ALKALIES, AND THE ISO-ELECTRIC POINT.

Solution of euglobulin by means of acid or alkali was shown by Hardy [1905] to be associated with the possession by the dispersed particles of an electric charge which was respectively positive or negative in sign.

Michaelis and Rona (1910, 2) found the iso-electric point of euglobulin to be at a concentration of hydrogen-ions equal to 36×10^{-7} normal and to coincide with the point of optimum flocculation for this protein.

The iso-electric point has been re-determined in the present instance and the result of Michaelis and Rona has been confirmed.

The euglobulin was prepared as follows: horse serum was diluted ten times with distilled water and the globulin was precipitated by acidifying with acetic acid (about 3-4 cc. N acetic acid per litre according to the original reaction of the serum). The precipitate was allowed to settle, was centrifuged off, and purified by dissolving in a minimal amount of standard sodium hydroxide solution (according to the amount of the precipitate) and reprecipitating with hydrochloric acid, the precipitate again being separated by centrifuging. This operation was repeated once or twice and the precipitate finally washed with distilled water. A fairly concentrated suspension was made in distilled water from which, by dilution, the material was prepared which was used for the various experiments¹. The particles of

¹ In some experiments, for example those in Tables IV and V, the euglobulin was prepared from horse-plasma (oxalate) by the method described by Mellanby [p. 339, 1905]. The purification in this case was just as above and no differences were detected between samples prepared by the two methods.

suspensions prepared as above invariably had a slight negative charge and the addition of a little acid was necessary in order to render the particles iso-electric with the solution.

In Table I is shown the degree of dispersion of a sample of euglobulin (0.032 % solution) corresponding with various concentrations of acid and alkali. The charge carried by the particles was at the same time determined

TABLE I.

Influence of reaction (hydrogen-ion concentration) upon the dispersion of euglobulin, and the electric charge carried by the protein particles; influence of sodium sulphate.

Concentration of protein = 0.032 %.							
Exp. No.	Cc. N/100 HCl (or equivalent) added in total volume of 10 cc.	Cc. N/100 NaOH (or equivalent) added in total volume of 10 cc.	Salt added	Concentration of salt, in terms of normality	Concentration of hydrogen-ions, in terms of normality	Sign of electric charge on the particles	Degree of agglutination
1	—	0.2	—	—	0.36×10^{-7}	—	Faintly opalescent soln.
2	—	0.1	—	—	0.97	—	Opalescent solution.
3	—	0.0	—	—	32	—	Agglutinated.
4	.1	—	—	—	748	—	Agglutinated later.
5	.15	—	—	—	1140	+	Opalescent solution.
6	.2	—	—	—	1390	+	Faintly opalescent soln.
7	.5	—	—	—	5080	—	Clear solution.
8	.2	—	Na ₂ SO ₄	0.03	0.97	—	Opalescent solution ¹ .
9	.5	—	„	0.05	1590	—	Opalescent solution ¹ .

¹ Agglutinated on standing.

by observing their behaviour in an electrical field, using the microscopic method previously employed by Martin and the author [1912, p. 285] in investigating the electrical properties of denaturated proteins. In the 5th column is given the concentration of hydrogen-ions in the various solutions, and the point of optimum agglutination is seen to be at a concentration equal to 32×10^{-7} normal. This figure is in good agreement with that found by Michaelis and Rona.

Confirmation of these values was incidentally obtained in the course of experiments made to elucidate other points. For example, the range of agglutination of another euglobulin suspension, containing 0.6 % protein was determined, after first dissolving in a minimum amount of dilute sodium hydroxide solution, by adding dilute hydrochloric acid to a series of tubes until precipitation occurred and finally dispersion was again obtained. Dispersion corresponded with a concentration of hydrogen-ions equal to 2.2×10^{-7}

normal on the alkaline side and 62.1×10^{-7} normal on the acid side. The solutions, of course, all contained a trace of salt.

In case of a third sample of euglobulin, see Table II (containing 0.016 % protein) the limits of agglutination were found to lie between concentrations of hydrogen-ions equal to 5×10^{-7} and 213×10^{-7} normal and precipitation to be rapid at a concentration of 18×10^{-7} normal.

TABLE II.

Influence of reaction (hydrogen-ion concentration) upon the dispersion of euglobulin and upon the electric charge carried by the protein particles; influence of sodium sulphate.

Concentration of protein = 0.016 %.

Exp. No.	Salt added	Concentration of salt added, in terms of normality	Cc. N/100 HCl (or equivalent) added in total volume of 10 cc.	Cc. N/100 NaOH (or equivalent) added in total volume of 10 cc.	Concentration of hydrogen-ions, in terms of normality	Sign of electric charge on particles	Degree of agglutination
1	—	—	0.3	—	—	+	Clear solution.
2	—	—	0.2	—	1280×10^{-7}	+	" "
3	—	—	0.1	—	213 "	+	Agglutination partial.
4	—	—	0.00	—	18.6 "	—	Agglutination complete.
5	—	—	—	0.1	5.41×10^{-7}	—	Agglutination partial.
6	—	—	—	0.2	—	—	Dispersed.
7	Na ₂ SO ₄	.02	0.5	—	1010×10^{-7}	—	Agglutination partial, less good than No. 8.
8	"	.03	"	—	137 "	—	Agglutination best, but not quite complete.
9	"	.04	"	—	65.9 "	—	Agglutination less good than No. 8.
10	"	.05	"	—	—	0	Dispersed.
11	"	.07	"	—	—	0	Dispersed.

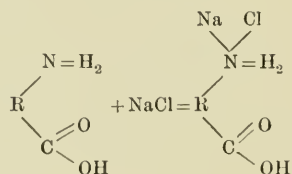
The experiments as to the amount and sign of the charge carried by the euglobulin particles, detailed in Tables I and II, are not calculated to define the iso-electric point with as great accuracy as those of Michaelis and Rona. As a rule the particles, while agglutinating rapidly, retained the negative charge which they originally held and only showed a positive charge when dispersion by acid was already well begun. Speaking generally, however, the point where the charge on the particles changed its sign coincided with the agglutination-zone. On the whole, there is a tendency to retain the negative charge rather than the positive. A distinctly positive charge was first detected on the globulin particles at a concentration of hydrogen-ions

equal to about 200×10^{-7} normal (see Exp. 3, Table II, and Exp. 2, Table VII).

The agglutination and dispersion of euglobulin by acids and alkalis, in confirmation of the results of Michaelis and Rona, is seen to be primarily dependent upon hydrogen-ion concentration, and in this respect a close analogy is presented with heat-denaturated proteins [Michaelis and Rona, 1910, 1; Sørensen and Jürgensen, 1911; Chick and Martin, 1912]. In both cases flocculation takes place when the protein particles are iso-electric with the solution, and, in cases where the reaction of the solution is more acid or alkaline than the iso-electric point, dispersion of the protein is due to the possession of a positive and negative electric charge respectively.

III. SOLUTION OF EUGLOBULIN BY NEUTRAL SALTS.

There has been some difference of opinion as to the mechanism involved in the solution of euglobulin by electrolytes. Hardy [1905] came to the conclusion that salt-solutions of globulin are without drift in an electric field and the particles of the system must be regarded as electrically neutral. He considered [1905, p. 325] "salt-globulin" to be the result of a molecular union of globulin and salt¹ by a process analogous to the formation of amino-acid-salt compounds², thus:



A similar theory had already been put forward by Pauli [1899].

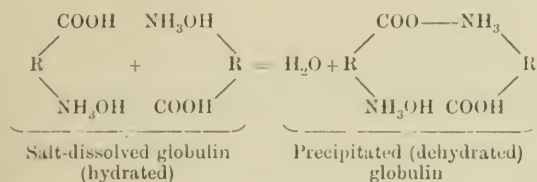
Mellanby [1905], on the other hand, has expressed the opinion that the solution of globulin by electrolytes is the result of activity of the constituent ions. He found that the efficiency of a salt in "dissolving" euglobulin depended on the valency of its ions, and, moreover, that the amount of globulin dissolved by a given concentration of any salt was proportional to the initial concentration of the protein. This curious fact was also noticed by Hardy [1905, p. 310].

A third view is that of Schryver [1910]. He considers (1) that, when precipitated, euglobulin is in a dehydrated condition, the amphoteric molecules having united by twos in salt formation with loss of the elements of

¹ Osborne and Harris [1905] also interpreted the solution of cdestin by certain electrolytes as due to the formation of such compounds.

² These appear to have been prepared in the crystalline form by Pfeiffer and Modelski [1912].

water and (2) that, when dissolved by electrolytes, the molecules remain separate in their hydrated condition, the aggregation in pairs being prevented by adsorption of the electrolytes on the surface of the molecules, thus



The insolubility of the dehydrated euglobulin Schryver attributes to the increased size of the molecule¹. There seems to be, however, better experimental evidence in support of the more usual conception that the euglobulin is precipitated in such circumstances as will allow of the aggregation of the protein particles under the influence of surface tension, that is to say, in the absence of other causes tending to keep them dispersed. Such causes are the presence of acid or alkali, in which case the euglobulin particles have been shown to be charged electrically, or the presence of electrolytes in whatever way the latter may act.

Schryver's view is in accord with that of Hardy in so far as the solution of euglobulin by electrolytes is attributed by both to the agency of the salt as a whole rather than to the activity of the constituent ions. His adsorption hypothesis, however, affords no explanation of the comparatively high concentration necessary for solution in case of many salts.

An additional theory, for which some experimental support is here given, is that dispersion of euglobulin by salts may be due to a specific adsorption by the protein particles of one—the more potent—ion of the electrolyte employed, and the acquisition of an electric charge by this means. Such an hypothesis would explain the influence of valency, demonstrated by Mellanby, and be consistent with what is known of the action of neutral salts in dispersing denaturated serum proteins [Chick and Martin, 1912, p. 281], with which euglobulin is in many ways analogous.

In the experiments detailed below, the "solution" of euglobulin by neutral salts was studied, using a series of electrolytes of more varied character, as regards the valency of the constituent ions, than those employed by the above observers. By this means some facts have been discovered which go to show that while, in some instances, solution of euglobulin by

¹ The conclusion of Dabrowski [1912] that in solutions of crystalline egg-albumin containing ammonium sulphate, the colloidal particles are of smaller size than in the case of the dialysed protein, is of interest in this connection.

neutral salts appears to be due to some action of the whole salt, in other cases it is undoubtedly associated with an acquirement of electric charge by the protein particles, the sign of which is determined by the more potent ion of the electrolyte employed.

1. *Experiments to determine whether salt-globulin is electrically charged.*

A series of experiments with a dilute englobulin suspension (0.016% protein) and a variety of salts is set forth in Table III. The object was to compare quantitatively the solvent and dispersing power of different ions and to decide whether solution was accompanied by the deposition of an electric charge upon the protein particles. The latter, in this instance, was determined by the microscopic method, using a dark ground and side illumination. The fluids were placed in a specially constructed cell and the behaviour of the particles in an electric field was observed. In using this method with fairly concentrated salt solutions (0.01 normal and upwards) there is some risk of disturbance by gas-bubbles given off at the electrodes, as the result of electrolysis. This has to be very carefully watched. As a rule the difficulty is minimised if the electrodes are previously platinised and the circuit closed only for the short time necessary to make the observation. The electromotive force employed was about 9 volts.

The results of this series of experiments show the relative solvent power of six different salts, viz. the chloride, sulphate and citrate of sodium, the chlorides of barium and calcium and lanthanum nitrate.

It is very clear that the influence of valency in preventing agglutination of englobulin is far from negligible, multivalent ions being much the more powerful in order of increasing valency. For example, the concentration of lanthanum nitrate and sodium citrate required for the purpose is respectively 1/30th and 1/60th of the necessary concentration of sodium chloride (5th column, Table III).

The electrical condition of the dispersed particles is set out in column 4, and it will be seen that, in four cases, a charge was demonstrated of similar sign to that carried by the more potent ion of the electrolyte employed. In the case of calcium¹ and barium salts, on the other hand, no charge could be detected on the protein particles which remained visible.

The *character* of the dispersion obtained was also different in the case of different salts, and could be grouped according to two types, which I have tentatively called the "electrical" and the "molecular" type of solution.

¹ This was also found to be the case when denaturated serum-proteins were dispersed by calcium salts [Chick and Martin, 1912, Table XIV].

TABLE III.

Solution of englobulin by electrolytes.

Concentration of protein = 0.016 %.

- × × × Complete agglutination, filtrate protein-free.
 × × Less complete agglutination, filtrate contained trace protein.
 × Partial agglutination.
 × - Almost complete dispersion.
 - Complete dispersion, clear solution.

Salt added	Concentration of salt, in terms of normality	Degree of agglutination	Sign of the electric charge upon the particles	Concentration of salt, in terms of normality, necessary	
				(a) to prevent agglutination	(b) to complete dispersion
NaCl	0.01	× × ×		0.03	—
	0.02	× × ×			
	0.03	× ×			
	0.04	× ×			
	0.045	× ×	- *		
	0.05	×	- *		
Na ₂ SO ₄	0.0005	× × ×		0.02	0.05
	0.001	× × ×			
	0.01	× × ×			
	0.02	× ×	- *		
	0.03	×			
	0.04	× -	- *		
	0.05	—			
Na ₃ Cit	0.0005	×	-	0.0005	0.003
	0.001	×			
	0.002	× -			
	0.003	—			
CaCl ₂	0.01	× × ×		0.02	—
	0.02	× ×			
	0.03	× ×			
	0.04	× ×			
	0.05	×	0		
BaCl ₂	0.001	× × ×		0.005	0.05
	0.005	× ×			
	0.008	× ×			
	0.01	× ×	0		
	0.02	×	0		
	0.03	× -			
	0.05	—	0		
La(NO ₃) ₃	0.001	× ×	+	0.001	0.02
	0.003	× ×			
	0.005	× ×			
	0.006	× -	+		
	0.008	× -			
	0.01	× -			
	0.02	—			

* Agglutinated particles.

That with a lanthanum salt or a citrate was exactly analogous to dispersion by acids and alkalis, the globulin suspension, with progressive increase in concentration of the salt, passed through various gradings of opalescent solutions until a clear transparent fluid was finally obtained. At the same time, under the microscope, using a dark ground illumination, innumerable particles could be seen which became smaller and smaller, acquiring at the same time an unmistakably positive or negative charge. Finally the whole field became a uniform grey in colour, until at length no particles could be distinguished.

In the case of the other salts used, there was usually no grading of opalescence, but, as the concentration of salt was increased, a decreasing amount of the original suspension remained undissolved in an otherwise clear fluid. Under the microscope there was a black and white effect, fewer and fewer agglutinated masses remaining visible on the black ground. This type of solution obtained with sodium sulphate and the chlorides of sodium, calcium and barium. In the case of the two latter salts, no charge was discovered on the visible particles; with sodium chloride and sulphate a negative charge was observed on the agglutinated particles which remained visible. It must however be remembered that the sign of the charge in both cases was negative, i.e. similar to that carried by the original globulin suspension.

The microscopic method is clearly unsuitable for studying the electrical condition of salt-globulin in such cases as the above, where "solution" is associated with a degree of dispersion which renders the dispersed particles immediately invisible. A series of corresponding experiments were accordingly made, in which the "U" tube method was employed, the object being to find out whether actual kataphoresis of the "salt-globulin" did or did not take place in an electric field.

This was the method used by Hardy [1905] whose conclusion that "salt-globulin" was without electric charge has already been referred to. In his experiments, however, as was pointed out by Michaelis [1909] the protein solution may not have been adequately protected from the influence of the acid and alkali produced at the electrodes by electrolysis of the salt present.

In order to avoid all complications due to electrolysis five U tubes were arranged in series in the present instance. The "salt globulin" occupied the centre tube, which was connected by a three-way tap on either side for convenience in filling. The other four tubes, two on either side, contained a solution of the same electrolyte in the same concentration as that used to disperse the euglobulin. All five tubes were fastened by brass clips to

a stand, so constructed that their position could be altered until the height of liquid in all the tubes was accurately adjusted to a standard level. Only after this was accomplished were the taps opened, and the euglobulin solution placed in contact with the other tubes. Control experiments showed that under these circumstances there was no transference of liquid from one tube to another. The electrodes were placed in the further arms of the two end tubes and the globulin solution was thus securely protected from the influence of any acid or alkali produced there by electrolysis. Litmus and phenol-phthalein were added to the tubes containing the positive and negative electrodes respectively and in all cases the experiment was discontinued long before there was any danger of acid or alkali reaching the centre tube. The resistance of this arrangement was very great, and, although the electrodes (small strips of platinum foil) were connected with the lighting circuit (200 volts) the current which passed amounted to only 0.0001 to 0.005 ampères according to the nature and concentration of the electrolyte employed. At the close of the experiment, which usually lasted from 5 to 10 hours, the contents of the nearer arms of the U tubes adjacent to the globulin tube were tested for the presence of protein by addition of Esbach's reagent or otherwise.

The results are set out in Table IV below, and, in general, confirm the results of Table III. In the case of calcium chloride, barium chloride and

TABLE IV.

*Electrical properties of euglobulin, dispersed by various electrolytes.
(U-tube method.)*

Protein content = 0.16 %.

Exp. No.	Salt	Concentration, in terms of normality	Electric field, volts.	Current, ampères	Duration of experiment, hours	Sign of electric charge upon protein	Appearance of solution	Sign of electric charge, observed by microscopic method
1	Na ₂ SO ₄	0.05	200	0.0025	8.5	0	Clear solution containing some particles undissolved.	- (agglutinated particles)
2	CaCl ₂	0.05	„	0.002	5.5	0	Almost clear solution, containing some particles.	0
3	BaCl ₂	0.04	„	0.005	5.0	0	Almost clear solution, faint opalescence.	0
4	La(NO ₃) ₃	0.005	„	0.0001	5.5	+	Opalescent solution	+
5	Na ₃ Cit	0.003	„	0.001	5.0	-	Opalescent solution	
6	„	0.05	„	0.0015	9.75	0	Clear solution containing some agglutinated particles.	

sodium sulphate in concentrations of 0.05, 0.04 and 0.05 normal respectively, no migration of the dissolved protein was demonstrated. With the last salt, however, the negative charge carried by the undissolved particles was again shown by the settling which took place less rapidly in the "positive" than in the "negative" arm of the centre U tube, showing that in the latter case the action of gravity was reinforced by motion of the particles towards the positive pole.

Englobulin dispersed by a small concentration of lanthanum nitrate (= 0.005 normal) displayed a marked positive charge and in the case of sodium citrate¹ a negative charge was shown under similar conditions (concentration = 0.003 normal). If the concentration of sodium citrate was increased to 0.05 normal, however, the dispersed englobulin showed no migration at all.

The case of sodium citrate is a very interesting one, as the character of the dispersion obtained by employing these two concentrations was also quite different, conforming respectively to the "electrical" and "molecular" types of solution described above (p. 324). With the dilute salt the dispersion is to an opalescent solution and is accompanied by the acquisition of a negative charge; in the stronger solution of citrate, the protein is uncharged and the liquid shows a small precipitate suspended in a clear fluid.

The change from the one type of solution to the other can be seen if a series of solutions be made up containing equal amounts of englobulin and a concentration of sodium citrate ranging from 0.001 to 0.05 normal. Dispersion is already well marked at 0.001 normal and continues through various grades of diminishing opalescence to a concentration of about 0.003 normal. At a concentration of 0.005 normal the opalescence is greater and at 0.01 normal, a distinct precipitate can be seen. In higher concentrations a gradual clearance takes place, but the solution is now of a different character, less and less of the precipitate remaining undissolved in an otherwise clear solution. In those experiments the protein content was 0.16 %.

Another interesting experiment is the following: The U-tube apparatus was arranged so that in the centre tube was a dispersion of englobulin in 0.003 N sodium citrate and in the side tubes 0.05 N sodium chloride. As the current passed a precipitation took place in the negative arm of the centre tube, the zone of which continually progressed towards the positive arm. This was followed by a zone of clear solution, also moving in the same

¹ These citrate solutions were carefully prepared to be quite neutral and the hydrogen ion concentration was approximately 10^{-7} normal.

direction. The interface between the advancing chlorine ions and the citrate ions will not be sharply defined, as the former travel more rapidly and tend to over-run and intermix with the latter. A possible explanation is therefore that as both citrate and chlorine ions move towards the positive pole, the latter replace the former in the solution around the protein which is also moving towards the positive electrode, but at a slower rate than either. In low concentration chlorine ions are unable to disperse globulin and precipitation occurs, to be followed again by solution when the concentration of the chlorine ions is sufficiently increased.

The conclusion to be drawn from these experiments is that solution of englobulin by neutral salts, in case of the more ordinary electrolytes, is due to the formation of some unionised and uncharged compound of salt and globulin—whether by a molecular union (Hardy) or as the result of adsorption (Schryver) there is not, at present, enough experimental evidence to decide. At the same time the phenomenon of dispersion by salts, at any rate in its beginning, is due to an electric charge being deposited on the particles of the englobulin suspension by the agency of the ions of the electrolyte. The influence of salts upon dispersions of englobulin by acids and alkalies, dealt with in the next section, is in support of this view.

In the case of the commoner salts, containing only mono- or divalent ions, the electric charges brought into play are not powerful enough to disperse the englobulin until the concentration is increased to a point where the second ("molecular") type of solution takes place. With such electrolytes the first or "electrical" type of solution as a rule is negligible. It was, however, detected in the case of sodium chloride and sodium sulphate. With sodium citrate, on the other hand, the one type was seen to give place to the other as the concentration of salt was progressively increased.

When englobulin is denaturated by heat, it loses its characteristic property of forming electrically neutral solutions with electrolytes. For example, after heating a dispersion of englobulin in 0.05 normal sodium sulphate, in which no kataphoresis of the protein could be demonstrated, the protein particles were found to migrate to the positive pole in an electric field. At the same time an alteration took place in the appearance of the solution—the degree of dispersion was diminished and a thick opalescence was developed. With dispersions in sodium chloride (0.1 normal) and calcium chloride (0.15 normal) almost complete agglutination took place on heating.

2. *Alteration in electrical conductivity during solution of euglobulin by electrolytes.*

It is clear that if a molecular union or an adsorption compound is formed during solution of euglobulin by electrolytes, there must be some diminution in electrical conductivity. Hardy [1905, p. 307] states that a loss of conductivity takes place equal to 1.4% to 2.4% in case of solution by magnesium sulphate and sodium chloride respectively.

The results of a special set of experiments, made for the purpose, confirm the result of Hardy.

The same sample of euglobulin was used as for the previous set of experiments, and the mixtures when prepared contained 0.6% protein. The conductivity was determined:

- (1) of the "salt-globulin" solution,
- (2) of an equal concentration euglobulin suspension in distilled water.

In the case of (1) and (2) the solutions were allowed to settle or were centrifuged and the conductivity measured in the supernatant liquid.

- (3) of an equal concentration salt solution in distilled water.

The conductivity of the distilled water used was found to be negligible in comparison; hence direct comparison was made between (1) and the sum of (2) and (3). All determinations were made at 18°.

The results are set out in Table V and show a loss of conductivity in every case investigated. The salts used were the chlorides of barium and sodium, sodium citrate and lanthanum nitrate. In the case of what I have termed the "electrical" type of dispersion the diminution in conductivity was proportionally greater than with the molecular type; in the former case it occurred with iso-electric euglobulin to the extent of 13.6% and 11.6% in case of dispersion by weak (0.004 normal) lanthanum nitrate and sodium citrate respectively, see Exp. 1. With more concentrated salt, 0.05 normal, the loss varied from 1-4% in the case of the four salts employed.

3. *Relation of the amount dissolved by a salt to the total amount of euglobulin present.*

Notice has already been made of the observation of Mellanby [1905, p. 342] confirmed by Hardy [1905, p. 310] that the amount of euglobulin dissolved by a given concentration of salt is, within certain limits, approximately proportional to the concentration of protein in the original suspension.

In the case of the salt used by Mellanby (sodium chloride in concentration

TABLE V.

Change in electrical conductivity on solution of euglobulin by electrolytes.

Concentration of protein = 0.6 %, temperature 18° C.

Concentration of protein—2.0 %; temperature 18° C.									Concentration of euglobulin in solution as "Salt-globulin," %
Exp. No.	Condition of euglobulin suspension	Salt	Concentration of salt, in terms of normality	Conductivity, in reciprocal ohms			Percentage loss in conductivity		
				Salt solution	Euglobulin suspension	"Salt-globulin" (found)		"Salt-globulin" (calculated)	
1	Approximately iso-electric	NaCl	0.05	0.1389	0.00050	0.1378	0.1394	1.17	—
		BaCl ₂	0.05	0.1368	0.00126	0.1368	0.1381	0.99	—
		Na ₃ Cit	0.004	0.005489	0.00046	0.005331	0.005949	11.6	—
		"	0.05	0.05289	0.00046	0.05236	0.05335	1.90	—
		La(NO ₃) ₃	0.004	0.006785	0.000345	0.006277	0.007130	13.6	—
		"	0.05	0.06708	"	0.06536	0.066742	3.14	—
2	Alkaline	Na ₃ Cit	0.004	0.005562	0.000569	0.005578	0.006131	9.93	0.28
		"	0.05	0.05357	"	0.05337	0.05414	1.45	0.41
		La(NO ₃) ₃	0.004	0.006845	"	0.006482	0.007414	14.4	0.34
		"	0.05	0.06681	"	0.06511	0.06738	3.5	0.51
3	Acid	Na ₃ Cit	0.004	0.005541	0.002145	0.006907	0.007686	11.3	0.07
		"	0.05	0.05359	"	0.05415	0.05573	2.93	0.42
		La(NO ₃) ₃	0.004	0.006850	"	0.008264	0.008995	8.85	0.45
		"	0.05	0.06794	"	0.06717	0.07008	4.33	0.53

from 0.04 to 0.09 normal) solution is of the "molecular" type. On the assumption that this solution is the result of a molecular union between salt and protein (Hardy) it is difficult to explain the existence of the above relationship. On the other hand, if Schryver's view of the salt-solution of euglobulin be accepted in one respect, that is to say if we consider the second or "molecular" type of globulin to be the result of "adsorption" of the salt as a whole, the amount of globulin dispersed by a given concentration of salt might be approximately proportional to the extent of adsorbing surface i.e. to the concentration of euglobulin¹.

It was also possible, however, that solution-rate might be conditioned by the *size* of the euglobulin particles (of which there would be every variety in such a suspension as that used by Mellanby) and that in the time of experiment, final equilibrium had not been maintained but only the particles of small size and comparatively large surface had been successfully attacked.

¹ The experimental support for this view is based upon the fact that the globulin-dissolving capacity and surface-tension (against air) of series of solutions of similar salts, were found to be inversely related to one another, which is the result that should obtain, in accordance with the Willard Gibbs hypothesis. The application of surface tension measurements of air against solution to the case of the protein against solution is not, however, without risk of error.

Some experiments were made to examine this theory, using equal volumes of a 3% suspension of globulin and 1/10th normal sodium chloride solution. It was found, however, that almost perfect equilibrium was attained within a few minutes after mixing. Experiments were also made, using in the one case finely divided euglobulin and in the other a similar suspension of globulin previously aggregated by freezing; the amount of protein dissolved in the second case by 0.05 normal sodium chloride, under similar conditions, was about 20% less than in the former. A phenomenon of this magnitude is, in itself, inadequate to explain the relationship observed by Mellanby and Hardy; it may, however, be a contributing factor.

The facts would appear to be best met by some such conception as the following: Solution of euglobulin by such electrolytes as sodium chloride or sodium sulphate, is due to the formation of a "soluble" compound of the globulin and the salt, which is electrically neutral, and to prevent the dissociation of which a large excess of the salt is necessary. Under such circumstances the final equilibrium, i.e. the relative proportion of "dissolved" and "undissolved" globulin, will largely depend upon the concentration of the salt employed and show an approximate constancy if the latter is maintained constant. In other words, the amount of dissolved euglobulin will be roughly proportional to the original concentration (Mellanby).

IV. THE EFFECT OF NEUTRAL SALTS UPON ACID AND ALKALINE DISPERSIONS OF EUGLOBULIN.

The influence of salts in causing precipitation of euglobulin previously dissolved in acid and alkali is analogous to the corresponding action with denaturated serum proteins.

Hardy [1905, p. 317] has drawn attention to the fact that the precipitating action¹ of a salt upon "acid or alkali-globulin" is due to one only of its ions, viz.: that which carries a charge opposite in sign to that carried by the protein, and that the higher the valency of this ion, the greater is the power of the salt.

These results have been confirmed by me and I have also been able to show that, if the concentration of salt is further increased, dispersion will again occur, the particles now taking a charge whose sign is determined by that of the more potent ion of the electrolyte employed. Thus the addition of an appropriate electrolyte to an alkaline or acid solution of euglobulin

¹ Mellanby [1905] has dealt with the precipitation of globulin from its solution in electrolytes. This is a different phenomenon and a high concentration of salt is required (salting out).

may, after first precipitating the protein, cause the particles to disperse again with a charge opposite in sign to that originally carried.

For example, an alkaline dispersion of euglobulin containing 0.016% protein, with the particles negatively charged, was precipitated by the addition of lanthanum nitrate to a concentration of 0.001 normal; at a concentration of 0.008 to 0.01 normal, dispersion again took place, the particles now being positively charged. In a similar experiment with sodium chloride the precipitation took place at a concentration of salt equal to 0.02 normal; at 0.05 normal the globulin was again dispersed, this time bearing a negative charge.

These results present a close analogy with what takes place in case of denaturated serum-proteins, where the degree of dispersion in acid and alkaline solution is also greatly influenced by the presence of neutral salts, and in two ways. In the first place the reaction of protein-containing solutions, whether denaturated or not, becomes altered on addition of neutral salts [Chick and Martin, 1911, p. 21; 1912, p. 280], being shifted in the direction of the neutral point. In acid solution, the concentration of hydrogen ions is lowered and the concentration of hydroxyl ions lessened if the solution be alkaline; the effect is related to the valency of the anion and kation respectively of the electrolytes in the two cases. In the second place, the electric charge carried by the protein particles is modified and may be lessened or even changed in sign by the addition of electrolytes if of opposite sense to that carried by the more potent ion of the electrolyte added.

The effect of the above-mentioned salts in modifying the reaction¹ of solutions containing protein has also been demonstrated in the case of euglobulin (see Tables I, VI and VII). It was therefore necessary to determine in how far the precipitating effect of salts was due to this effect. It is evident that solutions either too acid or too alkaline for precipitation of euglobulin might be adjusted to the iso-electric reaction by the addition of an appropriate electrolyte, and indeed this frequently occurred. For example (Experiment 12, Table VI), addition of sodium citrate to a concentration of 0.002 N, caused precipitation of an acid dispersion of euglobulin (0.016% protein) at the same time reducing the concentration of hydrogen ions to a point very near the iso-electric point for this protein. In some cases the change of reaction extended to the other side of the iso-electric point, e.g. Table VI, Experiment 13; in this case the observed change of sign in electric charge taking place simultaneously with dispersion by sodium citrate, could be explained on the ground of change of reaction alone. By comparison of

¹ The phenomenon in absence of protein is perceptible but negligible.

Experiment 7 with 12 and 13, Table VI, this change in reaction is seen to be increased proportionally with the degree of valency possessed by the ions of the salt employed.

TABLE VI.

The effect of sodium sulphate and citrate upon an acid dispersion of euglobulin (0.016 %).

Exp. No.	Salt added	Concentration of salt in terms of normality	Cc. N/100 HCl (or equivalent) added in total volume of 10 cc.	Concentration of hydrogen ions, in terms of normality	Sign of electric charge carried by the particles	Degree of agglutination
1	—	—	0.0		—	Partial agglutination.
2		—	0.075		—	Agglutinated completely.
3		—	0.1		—	" "
4		—	0.2	$10^{-4.10} (810 \times 10^{-7})$	+	Dispersed, clear solution.
5		—	0.3		+	" " "
6		—	0.7		+	" " "
7	Na ₂ SO ₄	0.03	0.5	$10^{-4.45} (357 \times 10^{-7})$	—	Agglutinated.
8		0.04	"		—	Partial agglutination.
9		0.05	"		—	Dispersed.
10		0.07	"		0	"
11	Na ₃ Cit	0.001	0.7		+	Dispersed.
12		0.002	"	$10^{-5.41} (39 \times 10^{-7})$	—	Almost complete agglutination.
13		0.003	"	$10^{-6.04} (9.2 \times 10^{-7})$	—	Dispersed.
14		0.004	"		—	Dispersed, faintly opalescent solution.

In many cases, however, acid and alkaline solutions of euglobulin, with their positively and negatively charged particles respectively, were first precipitated and afterwards dispersed, the particles bearing an electric charge of changed sign in solutions whose reaction still remained more acid and more alkaline respectively, than the iso-electric point¹. A good example of this is seen in Table I, Experiment 9, where addition of 0.05 N sodium sulphate to an acid dispersion of euglobulin (0.032 % protein) caused the particles to be dispersed and to carry a negative charge in a solution where the hydrogen ion concentration, equal to 1590×10^{-7} normal, was far on the acid side of the iso-electric point. Experiment 7 of Table II is another instance of the same phenomenon¹.

With an alkaline suspension of euglobulin a corresponding series of results was obtained with lanthanum nitrate. The results are set out in

¹ In higher concentration of sodium sulphate, the dispersed globulin appears to be electrically neutral, see Exp. 10, Table VI.

Table VII. A preliminary set of experiments (1 to 8), in absence of electrolytes, showed a positive charge to be acquired by the protein particles at a concentration of hydrogen ions equal to 204×10^{-7} normal. In the alkaline suspension used, the concentration of hydrogen ions was equal to 0.0013×10^{-7} normal. In presence of 0.006 normal lanthanum nitrate the

TABLE VII.

Effect of lanthanum nitrate upon an alkaline dispersion of euglobulin.

Protein = 0.016 %.

Exp. No.	Salt added	Concentration of salt, in terms of normality	Cc. N/100 HCl (or equivalent) added in a total volume of 10 cc.	Cc. N/100 NaOH (or equivalent) added in a total volume of 10 cc.	Concentration of hydrogen ions, in terms of normality	Sign of electric charge carried by the particles	Degree of agglutination
1	—	—	0.2	—			Dispersed, clear solution.
2	—	—	0.1	—	$10^{-4.69}$ (204×10^{-7})	+	Dispersed, faintly opalescent solution.
3	—	—	0.05	—	$10^{-7.00}$ (1.05×10^{-7})	—	Agglutinated.
4	—	—	0	—		—	Dispersed, opalescent sol.
5	—	—	—	0.5			" " "
6	—	—	—	0.1			Dispersed, clear solution.
7	—	—	—	0.2			" " "
8	—	—	—	0.5	$10^{-9.88}$ (0.0013×10^{-7})		" " "
9	La(NO ₃) ₃	0.0005	—	0.5			Dispersed, opalescent sol.
10		0.001	—	"			Agglutinated.
11		0.002	—	"		+	Agglutinated almost completely.
12		0.004	—	"		+	" " "
13		0.005	—	"		+	Agglutination not complete.
14		0.006	—	"	$10^{-7.63}$ (0.24×10^{-7})	+	" " "
15		0.008	—	"			Dispersed partly.
16		0.01	—	"			" " "
17		0.02	—	"			" " "

alkalinity was reduced almost to the neutral point. At the same time the charge on the particles was found to be positive in a solution whose reaction was on the alkaline side of the iso-electric point, and where, in the absence of any electrolyte, the particles would be negatively charged.

In these instances the effect of electrolytes recalls the exactly similar set of phenomena obtaining in the case of denaturated serum-proteins mentioned

above, and also the analogous influence of salts which has been observed in case of inorganic colloidal solutions [Burton, 1909]. In certain of the latter there has been demonstrated a selective adsorption of the ion bearing a charge opposite in sign to that of the charged colloidal particles [Linder and Picton, 1895; Whitney and Ober, 1902; Freundlich, 1910] and this explanation may be extended to the case of proteins.

In this connection, certain of the conductivity determinations in Table V, i.e., those of Experiments 2 and 3, are of special interest. These experiments were made with suspensions of euglobulin which were slightly on the acid and alkaline side, respectively, of the iso-electric point. The effect of adding electrolytes containing trivalent positive ($\text{La}(\text{NO}_3)_3$) and negative (Na_3Cit) ions was carefully studied. The loss of conductivity was found to be greater for sodium citrate if the globulin suspension were originally acid (i.e. charge on the protein particles positive) and greater for lanthanum nitrate if the globulin suspension were alkaline. At the same time, the degree of dispersion was measured by the content of protein in the supernatant fluid after centrifuging (see last column), and was found to be less. In all cases equivalent solutions of the salts were compared and the effects were more marked in the experiments with less concentrated salt (0.004 normal), in which case dispersion has been shown to be of the "electrical" type.

Analogy with euglobulin presented by caseinogen and other proteins.

Caseinogen has been found to show close analogy with euglobulin as regards the effects of electrolytes upon its solutions in either acid or alkali.

Michaelis and Rona [1910, 2] drew attention to the fact that among the naturally occurring proteins whose solution was accompanied by the acquisition of electric properties, in addition to globulin, were caseinogen, gliadin, and edestin, and they determined the iso-electric point in each case. The result was especially interesting in the case of caseinogen, where the particles were found to be iso-electric with the solution, and to be precipitated when the concentration of hydrogen ions was 1.8×10^{-5} normal, a degree of acidity far beyond that determined for euglobulin or for the heat-denatured proteins of serum.

The following experiments, set out in Table VIII, show the analogy with euglobulin to be closely maintained in respect also of the action of electrolytes. For example, in an opalescent solution (0.05 %) in weak hydrochloric acid, in which the protein particles were yet visible under the microscope using a high power and dark ground illumination, the caseinogen was found

to be positively electrified. This solution was readily precipitated by a minute concentration of sodium sulphate (equal to 0.0005 normal) or sodium citrate (0.00005 normal); with increased concentration of either salt (0.015 and 0.001 normal, respectively) the protein particles were again dispersed and found to carry a negative charge in both cases, see Table VIII, Experiment A.

TABLE VIII.

Influence of electrolytes upon agglutination of caseinogen
(Merck's pure casein).

(A) Dispersed with a little HCl.

(B) Dispersed with a little NaOH.

× × × Complete agglutination.

× Partial agglutination.

× × Almost complete agglutination.

× - Almost complete dispersion.

- Complete dispersion.

Exp. No.	Concentration of protein, %	Salt added	Concentration of salt, in terms of normality	Degree of agglutination	Sign of electric charge carried by the particles
(A)	0.05	0	0.00	-	+
	"	Na ₂ SO ₄	0.0005	× × ×	
			0.001	× ×	
			0.005	× ×	
			0.01	× -	
			0.015	-	-
			0.02	-	
	"	Na ₃ Cit	0.00001	× ×	
			0.00005	× × ×	
			0.0001	× ×	
			0.0005	×	
			0.001	-	-
			0.002	-	
(B)	0.03	-	0.00	-	-
	"	CaCl ₂	0.01	-	
			0.02	× -	-
			0.05	×	-
			0.09	×	0
			0.10	×	
			0.15	×	
			0.2	× -	0
			0.5	-	
	"	La(NO ₃) ₃	0.00002	-	
			0.00005	×	-
			0.00008	×	
			0.0001	× ×	
			0.0002	× × ×	
			0.0005	× -	
			0.001	-	+

Experiment (B) with an alkaline dispersion of caseinogen 0.03%, showed an exactly analogous set of phenomena with lanthanum nitrate.

With calcium salts the action appears to be different in character. In the first place no complete precipitation takes place; the size of the particles is increased, but no complete agglutination occurs although the solution becomes turbid. The particles, when dispersed again by increased concentration of the salt (0.2 to 0.5 normal) do not appear to carry any electric charge. In this respect also the analogy with both denaturated serum-proteins and with euglobulin is maintained.

V. SUMMARY.

1. The iso-electric point for euglobulin has been re-determined and found to coincide with the point of most rapid agglutination, viz.: at a hydrogen ion concentration of about 3×10^{-6} normal, a figure which agrees with that obtained by Michaelis and Rona [1910, 2].

2. The solution or dispersion of euglobulin by electrolytes is shown to be much influenced by the nature (especially as regards valency) of the constituent ions and to be of two general types:

(a) "electrical" type of solution in which the euglobulin dispersion is accompanied by the acquisition of an electric charge by the protein particles, the sign of which is similar to that of the more potent ion of the electrolyte employed.

(b) "molecular" type of solution, in which the dissolved euglobulin is electrically neutral.

In type (a) the dispersion is considered to result from a specific adsorption of the ion possessing the higher valency, in (b) from a molecular union with (Hardy) or adsorption of (Schryver) the salt as a whole. Both types of solution are accompanied by loss of electrical conductivity in the liquid.

The "electrical" type of solution is well seen in case of dispersion by such salts as sodium citrate and lanthanum nitrate in low concentration; in case of the more ordinary salts, containing mono- or divalent ions only, the electric forces concerned are not powerful enough to disperse globulin until the concentration is raised to a point where "molecular" solution takes place. In the case of sodium citrate, the "electrical" type of solution was found to change to the "molecular" type as the concentration of the salt was increased.

3. Euglobulin, when denaturated by heat, no longer possesses the property of forming the "molecular" type of solution with electrolytes. On

heating the latter, in some cases the degree of dispersion is merely diminished, and the protein particles acquire an electric charge, whose sign is determined by the more potent ion of the electrolyte employed; in other cases agglutination takes place.

4. The reaction of acid and alkaline solutions of euglobulin is greatly influenced by the addition of electrolytes, the hydrogen and hydroxyl ion concentration being reduced respectively. In case of the former the effect is much increased with rising valency of the anion and in alkaline solution the result is determined by the valency of the kation.

5. The influence of electrolytes in causing precipitation of globulin dissolved in acid and alkali may, in some instances, be adequately explained by the alteration in reaction, described under 4; in this way solutions too acid or too alkaline for agglutination of the globulin may be adjusted to the iso-electric point by the addition of an appropriate electrolyte.

Precipitation by electrolytes may, however, also take place in solutions whose reaction is still far removed from that of the iso-electric point. In these instances it is attributed to neutralisation of the electric charge originally carried by the protein particles by means of a specific adsorption of the oppositely charged ion of the electrolyte; the effect is related to valency.

6. In the properties regarding solution and precipitation detailed under 1, 4 and 5, euglobulin, in common with caseinogen, and the vegetable globulins presents a very interesting analogy with heat-denaturated proteins. Euglobulin differs from heat-denaturated protein in its capacity to form solutions with electrolytes in which the protein particles are electrically neutral.

In conclusion, I wish to express my indebtedness to Prof. C. J. Martin for much helpful advice and criticism.

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XXXIV. THE VISCOSITY OF PROTEIN SOLUTIONS. II. PSEUDOGLOBULIN AND EUGLOBULIN (HORSE).

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(Received May 18th, 1914.)

The following experiments form a continuation of those published recently by Lubrzynska and the author [1914], on the influence of concentration of protein upon the viscosity of horse serum and solutions of crystallised egg and serum albumin (horse). In the present case the investigation has been extended to the pseudoglobulin and euglobulin of horse serum. The significance of both sets of results is discussed at the end of the present paper.

PREPARATION OF THE MATERIAL.

By pseudoglobulin is signified the protein, soluble in distilled water at its iso-electric point, which is separated from serum by one-half saturation with ammonium sulphate¹ (addition of an equal volume of saturated ammonium sulphate solution to the diluted serum). The term euglobulin is used to indicate the protein insoluble in distilled water at its iso-electric point and therefore precipitated by dilution of the serum and acidification until the reaction is adjusted to the iso-electric point of the protein.

The protein precipitated by one-third saturation with ammonium sulphate (addition of one volume of saturated ammonium sulphate to two volumes diluted serum) is frequently considered to be euglobulin, and this method is a standard one for separating this protein from pseudoglobulin. The precipitate obtained, however, is a mixture of the two, and the total amount is considerably more than is obtained by dilution and acidification. Further, it does not include all the euglobulin, for the precipitate of pseudoglobulin, obtained when the concentration of ammonium sulphate in the filtrate is

¹ There is no reason to suppose that this method sharply separates pseudoglobulin from albumin [see Chick and Martin, 1913].

raised to one-half saturation, is found to contain more protein insoluble in water (euglobulin), than if the latter had previously been removed by dilution and acidification.

Euglobulin. In the present case the euglobulin was always prepared by the last named method. Serum was diluted ten times and the reaction adjusted to the iso-electric point of the euglobulin by means of the addition of a small amount of acetic acid, about 2-3 cc. normal acetic acid being, as a rule, found necessary for each litre of diluted serum. The optimum amount was determined by making preliminary trials with small samples, and was easily recognised by the ease and rapidity with which the precipitate settled. After one to two days' standing the precipitate was separated by centrifuging the deposit, after decanting the top liquor. It was purified three or four times by redissolving (dispersing) in a minimum of caustic soda and precipitating with an exact equivalent of hydrochloric acid. The stronger acid is here employed in place of acetic, because a smaller quantity is found to be necessary and a lower concentration of salt results on neutralising. The final precipitate was washed once with slightly acidified water. For this purpose water, saturated with carbon dioxide and diluted about 30 times with ordinary distilled water, has been found to yield the convenient, slight, acidity.

Pseudoglobulin. The pseudoglobulin, used for the experiment given in Table V, was prepared from diluted horse serum by one-half saturation with ammonium sulphate, after removal of the precipitate given by one-third saturation. The crude precipitate was dissolved in water and twice reprecipitated, the final material being dialysed for two days against running water (tap) and for sixteen days against distilled water, changed daily, in presence of toluene.

The method of precipitation by one-third saturated ammonium sulphate, as stated above, does not remove all the euglobulin. The small proportion remaining can be detected even before the dialysis is commenced, by diluting the material and acidifying with minute amounts of dilute acetic acid, a method by which euglobulin can readily be detected in presence of a small concentration of salt [see Chick, 1913]. After dialysis for nine days, as much as one-tenth of the total protein was found to be insoluble in distilled water. At the completion of the process the material contained about 14% protein, of which about one-eighth was found to be in this condition. This insoluble material, the presence of which must, I think, be attributed to a gradual degradation of the pseudoglobulin to a water-insoluble condition, shows very remarkable analogies with euglobulin and has been made the

subject of a separate investigation, the result of which will be published later. When the dialysis was complete¹, the insoluble material could be completely separated by diluting and centrifuging. The top liquor was found to be free from insoluble protein and was concentrated over sulphuric acid in a vacuum in order to obtain material of the right strength for the viscosity measurements.

In order to investigate material which had suffered no degradation, dialysis was dispensed with in preparing that used for the experiments in Table VI. The euglobulin was removed in the ordinary way from the serum by dilution (1 in 10) and acidification and a further precipitate separated by addition of ammonium sulphate to one-third saturation (19.8 g. $(\text{NH}_4)_2\text{SO}_4$ to 100 cc.); ammonium sulphate was then added to the filtrate until one-half saturation was reached (11.3 g. $(\text{NH}_4)_2\text{SO}_4$ to 100 cc., final density 1.138). The pseudoglobulin was purified as follows. The precipitate was pressed between filter paper to free it from mother liquor, redissolved in water and an equal volume of saturated ammonium sulphate was added. The resulting mixture contained an excess of ammonium sulphate, over one-half saturation, owing to the salt contained in the precipitate. This extra amount was ascertained by boiling a small sample of the mixture, filtering the protein and determining the density of the filtrate. The requisite amount of water could be then calculated and added to the mixture. The precipitate was filtered, freed from mother liquor as far as possible by pressing between filter paper and finally dissolved in a little water. The solution was found to have the following composition: protein 13.75 %, ammonium sulphate 10.46 %. It contained only the faintest trace of insoluble protein (tested by dilution and acidification).

EUGLOBULIN.

Influence of concentration of protein.

The experiment, of which the results are detailed in Table I, was so arranged that the conditions of solution (dispersion) should approximate to those obtaining in normal serum. The euglobulin was dispersed with a small amount of alkali, the concentration of hydrogen ions, 10^{-8} N, approximated to that of serum, and in addition a small amount of sodium chloride was present, about 1 gram per 6 grams protein. The results are shown graphically

¹ During the dialysis of proteins, the reaction gradually approximates to the slight acidity characteristic of the iso-electric point, and as a consequence any euglobulin or "denaturated" protein is gradually precipitated.

in curve *c*, Figure 1, where the results of similar experiments with pseudoglobulin are also plotted, together with those for serum albumin and whole serum [see Chick and Lubrzynska, 1914, p. 61] for purposes of comparison. The much greater viscosity of euglobulin is at once apparent. A solution containing 6.6 % protein has a viscosity of 3.49, i.e. more than twice that shown by solutions of serum albumin or by whole serum of equal protein-content. With higher concentration of protein (12.95 %), the viscosity of euglobulin reaches the high figure of 21.7, a value not approached by the strongest solutions obtained of serum albumin (20.6 % protein, coefficient of viscosity 7.54) or of the proteins of whole serum (18.1 % protein, coefficient of viscosity 6.38).

TABLE I.

Influence of concentration of the protein upon the viscosity of solutions (dispersions) of euglobulin (horse) in dilute sodium chloride solution and alkali.

* Concentration of sodium chloride 2.2 % to 0.5 %.

* „ „ „ hydrogen ions $10^{-8.0}$ to $10^{-8.2}$ normal.

Temperature 25°. Time of flow in viscosimeter for water = 18.5 seconds.

Concentration of protein, %	Mean time of flow in viscosimeter, secs.	Density of solution (H ₂ O at 25° = 1)	Coefficient of viscosity, H ₂ O = 1
12.95	379.3	1.0582	21.69
10.81	191.7	1.0479†	10.86
9.84	144.9	1.0435	8.17
6.60	62.7	1.0290	3.49
3.27	31.2	1.0141	1.71

* Concentration of salt and alkali was adjusted to give results comparable with those obtained for whole horse serum [see Chick and Lubrzynska, 1914, Table VI, p. 67].

† Interpolated value.

Influence of hydrogen ion concentration and of salt content.

Hardy [1905] found that the viscosity of euglobulin, when dissolved by salt, was much lower than when dispersed by alkali. When small amounts of alkali are used, this is undoubtedly the case, the opalescent material obtained being much more viscous than the clear solution formed when salt is used to dissolve the protein. If, however, the concentration of alkali is increased, the viscosity falls rapidly. This is seen in Table II, where, in Experiment I, the result is given of three determinations of viscosity in solutions containing 5.6 % protein, but with concentration of hydrogen-ions falling from $10^{-7.46}$ N to $10^{-10.02}$ N. Corresponding to this change in reaction, there is a fall in the coefficient of viscosity from 12.17 to 3.62. The last

result is not greatly in excess of that obtained in Experiments II and III which deal with solutions of salt-globulin of the same protein concentration. The reaction in these two instances is not far removed from that of the iso-electric point which was determined by Michaelis and Rona [1910] to be at a concentration of hydrogen-ions equal to about 10^{-6} N. The direct influence of salt in lowering viscosity¹ is seen by a comparison of Experiment IV with Experiment I. In the former case, a small concentration of salt, 1 %, reduced the coefficient of viscosity from about 12.2 to 2.4 in presence of, roughly, the same concentration of hydrogen-ions. The figures for Experiments II and IV are interpolated values obtained from curves *b* and *c* respectively, in Fig. 1.

TABLE II.

Influence of concentration of hydrogen-ions upon the viscosity of euglobulin (horse) (a) dispersed by alkali, Exp. I; (b) by NaCl, Exps. II and III, and (c) by alkali + NaCl, Exp. IV.

Concentration of protein 5.68 %. Temperature 25°.					
Exp.	Concentration of NaCl, %	Concentration of alkali, NaOH, in terms of normality	Concentration of hydrogen-ions, in terms of normality	Density of the solution (H ₂ O at 25° = 1)	Coefficient of viscosity (H ₂ O, or salt solution, = 1)
I	—	—	$10^{-7.46}$	1.0164	12.17
	—	0.01	$10^{-9.34}$,,	6.84
	—	0.02	$10^{-10.02}$,,	3.62
II	3.6	—	$10^{-5.7}$	—	2.65*
III	3.5	—	$10^{-6.2}$	—	2.77†
IV	1.0	—	$10^{-8.1}$	—	2.39*

* Interpolated values from curves *b* and *c*, Fig. 1, see also Table I and Table III (Exp. II).

† See Exp. I, Table III.

In Table III are given the results of two experiments showing the influence of protein concentration when the euglobulin, at or about its iso-electric point, is dispersed by means of salt alone. It is seen that, when the concentration of protein is sufficiently high, very high values are obtained for the coefficient of viscosity. For example, in Experiment II, when the protein concentration was 13.2 %, the coefficient of viscosity was 29.56.

From a comparison of Tables II and III with Table I, which deals with an experiment in which the conditions obtaining in serum were closely imitated, it is evident that the euglobulin in serum must be regarded as salt-globulin.

¹ This influence would appear even more marked if, in calculating the concentration of protein in the solutions of salt-globulin, any allowance were made for the water appropriated by the salt.

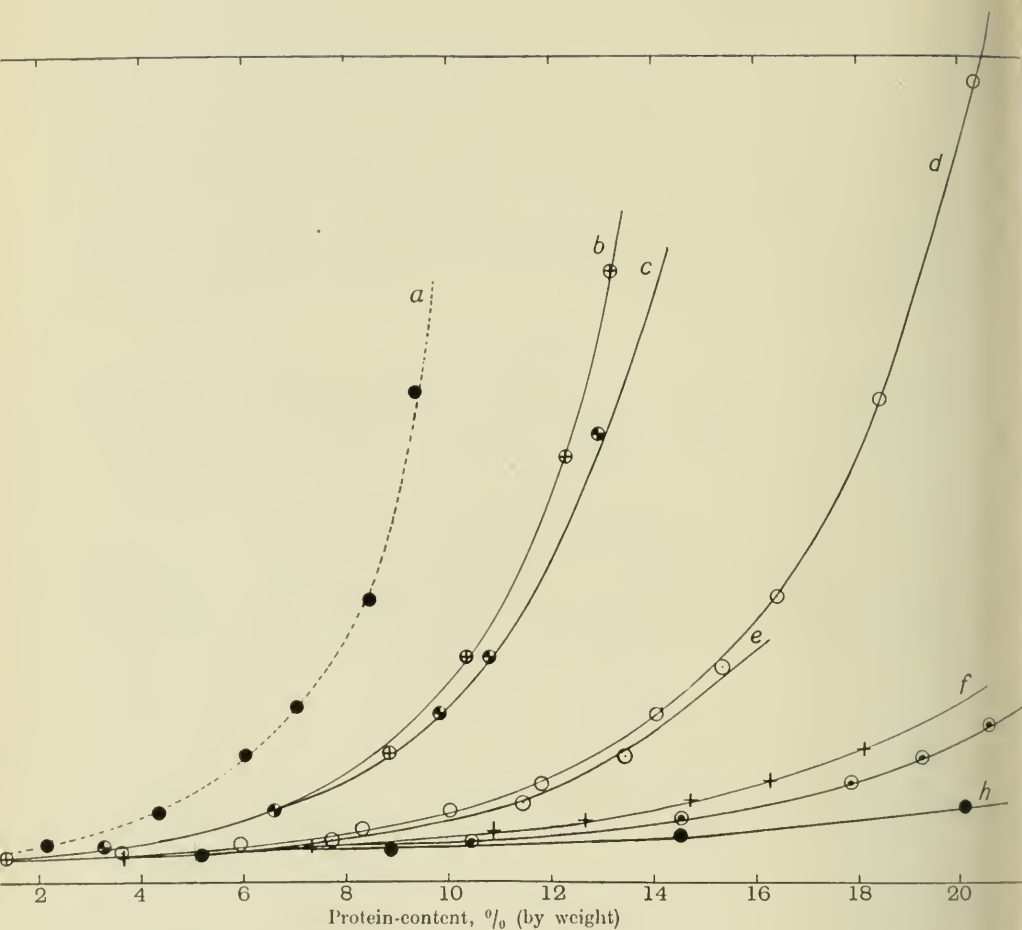


Fig. 1. Influence of protein concentration upon the viscosity of various protein solutions.

- Curve *a* -----●-----, sodium caseinogenate [Chick and Martin, 1913].
 „ *b* ———⊕———, englobulin (salt). (See Exp. II, Table III.)
 „ *c* ———●———, englobulin (salt + alkali). (See Table I.)
 „ *d* ———○———, pseudoglobulin. (See Table V.)
 „ *e* ———⊖———, pseudoglobulin (salt). (See Table VI.)
 „ *f* ———+———, whole serum (horse). [Chick and Lubrzenska, 1914, Table VI.]
 „ *g* ———⊙———, serum albumin. [„ „ „ „ „ IV.]
 „ *h* ———●———, egg albumin. [„ „ „ „ „ I.]

TABLE III.

Influence of concentration of protein upon the viscosity of euglobulin (horse) dissolved (dispersed) in sodium chloride solutions.

Exp. I. Concentration of hydrogen-ions = $10^{-6.2}$ normal.

Exp. II. " " " = $10^{-5.5}$ to $10^{-6.0}$ normal.

Temperature = 25° .

Mean time of flow for water = 55.4 seconds.

Exp.	Concentration of NaCl, %	Concentration of protein, %	Mean time of flow in viscosimeter, secs.		Density (H_2O at $25^{\circ}=1$)		Coefficient of viscosity, H_2O containing 3% NaCl = 1
			Euglobulin solution	Salt solution (3.0% NaCl)	Euglobulin solution	Salt solution (3.0% NaCl)	
I	3.0	8.02	241.1	57.6	1.045	1.0211	4.28
	3.7	5.82	142.3	"	1.038*	"	2.48
	4.3	3.62	95.2	"	1.032	"	1.62
	3.5	1.44	68.6	"	1.025	"	1.18
II	3.6	13.20	1654.1	(3.6% NaCl) 58.1	1.0650	(3.6% NaCl) 1.0257	H_2O containing 3.6% NaCl = 1 29.56
	"	12.32	1153.3	"	1.0626*	"	20.56
	"	10.57	612.3	"	1.0574*	"	10.87
	"	8.86	352.8	"	1.0522*	"	6.23
	"	6.58	189.4	"	1.0451	"	3.32
	"	1.36	69.1	"	1.0298*	"	1.19

* Interpolated values.

Influence of Temperature.

The solution used to investigate the influence of temperature contained 10.81 % protein and 1.82 % sodium chloride, and had a hydrogen-ion concentration equal to 10^{-8} N, the conditions being so arranged as to approximate to those obtaining in normal serum. The viscosity at various temperatures is expressed in relation to that of a 1.82 % solution of sodium chloride, estimations of the latter being made in the same viscosimeter at points over the same range of temperature. The results were expressed in a smoothed curve, from which the values in the 3rd column of Table IV were obtained. With a rise of temperature from 21° to 41.3° , the coefficient of viscosity was decreased to less than one quarter (see Table IV), an effect very much greater than that obtained with the other serum proteins investigated. A solution of serum albumin containing 20.65 % protein had its viscosity reduced by only about 30 % for a similar change of temperature, and while, for the whole proteins of serum, the effect of temperature was somewhat greater, it was insignificant compared to the result obtained for euglobulin.

TABLE IV.

Influence of temperature upon the viscosity of solutions (dispersions) of euglobulin (horse) in dilute sodium chloride and alkali.

Concentration of sodium chloride = 1.82 %.			
,, ,, hydrogen-ions = $10^{-8.03}$ normal.			
,, ,, protein = 10.81 %.			
Density of protein solution = 1.0479.			
,, ,, 1.82 % NaCl solution = 1.0127.			
Temperature, °C.	Mean time of flow in viscosimeter, secs.		Coefficient of viscosity (1.82 % NaCl solution at the same temperature = 1)
	Euglobulin solution	1.82 % solution of NaCl (from curve)	
2.1	939.8	36.3	26.79
9.1	532.4	29.5	18.67
17.4	288.7	24.1	12.39
25.0	198.1	20.8	9.85
32.1	138.6	18.6	7.71
41.3	93.6	16.2	5.97

The great influence of rise of temperature in lowering viscosity is characteristic of emulsoid colloids, perhaps the most striking example being that of solutions of gelatin. The difficulty experienced in filtering serum and other solutions containing proteins is also lessened to a surprising extent on raising the temperature owing to the fall in viscosity. With dilute solutions, of low viscosity, the effect may not be much in excess of that observed for water itself, or for solutions of crystalloids, but it becomes progressively greater with increase in concentration of the colloids and consequent rise in viscosity. For example, a solution of egg-albumin containing 7.04 % protein (coefficient of viscosity = 1.36 at 25°) showed a change of viscosity only about 5 % greater than that of distilled water when the temperature was raised nearly 40 degrees centigrade. When the strength of protein was increased to 20.1 % (coefficient of viscosity = 3.58 at 25.4°) and 28.15 % (coefficient of viscosity at 25.4° = 10.01) the change in the value of the coefficient was about 23 % and 54 % respectively for a similar change in temperature [see Chick and Lubrzenska, 1914, Table II]. In the case of whole serum (protein = 7.68 %; coefficient of viscosity = 1.87 at 25°), the coefficient of viscosity was decreased nearly 14 %, and with concentrated serum (18.1 % protein, coefficient of viscosity = 6.34 at 25°) the fall in value was about 51 % on raising the temperature about 40° [Chick and Lubrzenska, 1914, Table VII].

It is therefore quite consistent that solutions of pure euglobulin, with their very high viscosity, should suffer an even greater change in this respect with alteration of temperature. An interpretation of the phenomenon will be suggested later (see p. 277).

PSEUDOGLOBULIN.

Influence of concentration of protein.

The results of viscosity measurements with solutions of pure dialysed pseudoglobulin are set forth in Table V. The method by means of which it was freed from the water-insoluble material, formed during its lengthy preparation, has already been described (p. 263). The results are also graphically shown in curve *d* of Figure 1. It will be seen that, as regards viscosity, solutions of pseudoglobulin occupy a position intermediate between those of euglobulin and of serum albumin. For solutions of equal protein content, the viscosity of those of pseudoglobulin falls far below those of euglobulin and this is especially marked in the stronger solutions. For example, a solution containing 10 % protein, exhibits a viscosity less than one-third of that of a solution of euglobulin of equal strength. At the same time, the viscosity is considerably in excess of that of serum albumin, a 10 % solution being nearly twice and a 20 % solution about five times as viscous [see Chick and Lubrzenska, 1914, p. 65].

TABLE V.

Influence of concentration of the protein upon the viscosity of solutions of pseudoglobulin (horse) dialysed.

Temperature 25°.

Time of flow in viscosimeter for water=48 secs.

Concentration of hydrogen-ions= $10^{-6.8}$ to $10^{-6.9}$ normal.

Concentration of protein, %	Mean time of flow in viscosimeter, secs.	Density of the solution (H ₂ O at 25°=1)	Coefficient of viscosity (H ₂ O=1)
20.37	1753.6	1.0619	38.79
18.45	1059.7	1.0558*	23.31
16.43	628.8	1.0490	13.74
14.06	370.8	1.0425	8.05
11.82	218.0	1.0341	4.70
10.04	160.1	1.0289*	3.43
8.32	121.5	1.0239*	2.59
5.95	87.5	1.0169	1.85
3.61	66.4	1.0102	1.40

* Interpolated values.

Influence of ammonium sulphate.

In Table VI are given the results of an experiment with un-dialysed material containing the salt which separated with the protein phase during precipitation by half-saturated ammonium sulphate. The insoluble protein present amounted only to a slight trace. The concentration of ammonium sulphate was equal to 10.4 %, and, in order to compare the results with those given in Table IV, the percentage concentration of protein is reckoned, not as grams per 100 grams of the system but as grams per 100 grams protein and water, see 3rd column Table VI.

TABLE VI.

Influence of concentration of protein upon the viscosity of solutions of pseudoglobulin (horse) containing ammonium sulphate.

Temperature 25°.

Time of flow in viscosimeter for 10.3 % $(\text{NH}_4)_2\text{SO}_4$ = 53.9 seconds.

Time of flow for water = 48 seconds.

Concentration of hydrogen-ions = $10^{-6.8}$ normal.

Concentration of $(\text{NH}_4)_2\text{SO}_4$ = 10.4 %.

Concentration of protein		Mean time of flow in viscosimeter, secs.	Density of solution (H_2O at 25° = 1)	Density of H_2O containing 10.3 % $(\text{NH}_4)_2\text{SO}_4$	Coefficient of viscosity, H_2O containing 10.3 % $(\text{NH}_4)_2\text{SO}_4$ = 1
G. in 100 g. total system	G. in 100 g. water + protein				
13.75	15.35	535.2	1.1025	1.0601	10.33
12.05	13.45	312.6	1.0972*	„	6.00
10.26	11.45	198.8	1.0917*	„	3.80
6.93	7.73	106.8	1.0814	„	2.02
4.16	4.65	75.3	1.0729*	„	1.41

* Interpolated values.

As a matter of fact, for accurate comparison, some allowance should also be made for the water appropriated by so large a proportion of salt, but that is impossible to estimate. While the effect of salt in lowering viscosity is much less than that obtaining in the case of euglobulin, its influence is evident from a comparison of Tables V and VI, or of curves *d* and *e*, Figure 1.

SIGNIFICANCE AND INTERPRETATION OF RESULTS.

From a survey of the foregoing results, it is seen that, as regards their viscosity, solutions of the three proteins of serum, albumin, pseudoglobulin and euglobulin, form a series varying from comparatively labile fluids, resembling those of crystalloids, in case of albumin, to liquids of considerable

viscosity, in the case of euglobulin. A concentration of protein of at least 10 % is necessary to reveal the colloidal nature of serum albumin and leads to excessively high viscosities with the other two proteins.

The interpretation of the characteristic phenomena displayed by solutions of these proteins may be found in the two-phase nature of the system. Proteins are "hydrophile emulsoids" and the actual volume of the disperse phase may be assumed to be much greater¹ than that indicated by the solution volume of the protein, as determined from the density of the solution.

Hatschek [1910, 1911] has developed a theory of the viscosity of two-phase systems based upon his own observations of the viscosity of oil-water emulsions. He has shown that when the volume of the oil approaches 70 % of the total volume, at which point the oil particles touch one another, there is an enormous increase in viscosity. This, in his opinion, is due to the inability of the oil particles to roll upon one another under the influence of a shearing force; as a result they suffer deformation. From mechanical considerations he has found that, when the volume of the oil is more than one-half the total volume, the viscosity of such a two-phase system can be expressed in terms of the phase ratio as follows:

$$\eta = \eta' \sqrt[3]{\frac{A}{A-1}} \dots\dots\dots(1),$$

where

η = the viscosity of the system,

η' = the viscosity of the continuous phase,

V = volume of the system,

v = volume of the disperse phase,

and

$A = \frac{V}{v}$ = the phase ratio.

If the viscosity of the continuous phase be taken equal to 1.0

$$\eta = \sqrt[3]{\frac{A}{A-1}} \dots\dots\dots(2),$$

or

$$A = \left(\frac{\eta}{\eta-1} \right)^3 \dots\dots\dots(3).$$

According to this expression, the viscosity of the system is independent of the viscosity of the disperse phase and of the size of its particles, but depends upon the relative volume of the two phases.

The formula was tested by Hatschek [1911, 1913] in the case of oil-water emulsions, of which the composition was accurately known. When

¹ In this connection, it is of interest to note that Findlay and Creighton [1911] found that the solubility of oxygen, at atmospheric pressure, in de-aerated serum was only about one-fifth as great as in water; in the case of nitrogen, the difference was even greater.

the emulsion was sufficiently concentrated, the known volume of oil emulsified was found to be in close agreement with the value calculated by means of the above formula.

Hatschek [1912] has found the above formula also applicable to colloidal solutions of the emulsoid type, in cases where the disperse phase occupies more than one-half the total volume of the system, i.e. where A is less than 2. The two instances selected by him are solutions of glycogen, using the viscosity measurements of Bottazzi and d'Errico [1906], and those of caseinogen, using the results of Chick and Martin [1912]. The value of $A \left(= \frac{V}{v} \right)$ was reckoned by means of the formula (3) given above, V being the volume occupied by 100 grams of the system. The value of $A' \left(= \frac{V}{c} \right)$, where c is the weight of dissolved substance, could be calculated from the measured density of the system and the known concentration of the colloid. The ratio $\frac{A'}{A} \left(= \frac{v}{c} \right)$, expressing the volume occupied by unit weight of the dissolved substance, was then calculated and, in case both of glycogen and caseinogen, Hatschek found a very fair constancy in value, when A was less than 2.

One of the most important results of Hatschek's expression is that, given the viscosity, not only is a method afforded of determining the phase ratio, but from the latter, given the concentration of the colloid and the density of the system, a calculation can, for the first time, be made of (a) the volume occupied, and (b) the amount of water taken up, by unit weight of a hydrophile colloid.

In the case of caseinogen, the value of (a) is equal to 9.3¹, that is to say each gram of caseinogen in colloidal solution occupies a volume equal to 9.3 cc. having taken up 8.6 cc. water (solution volume of caseinogen = 0.70). It follows therefore that even in so comparatively dilute a solution as 6% (100 grams of the system occupying 98 cc.) the caseinogen phase would occupy 56 cc. or 57% of the total volume.

Using Hatschek's formula the values of $\frac{v}{c}$ for all the serum proteins used in the present investigation have been calculated and the results for englobulin and pseudoglobulin are given in Tables VII and VIII and IX respectively. Table X contains a similar set of results for serum albumin, using the viscosity measurements published previously [Chick and Lubrzynska, 1913].

¹ The mean value given by Hatschek, 9.52, is slightly too high, the density of the system being neglected in evaluating A' .

TABLE VII.

Volume occupied by the euglobulin phase in solution at 25° C.

(a) Dispersed with salt and alkali (see Table I).

(b) Dispersed with alkali (see Table II).

Concentration of protein, % = c	Concentration of hydrogen-ions, in terms of normality	Concentration of NaCl, % = δ	Density of system = δ	Coeff. of viscosity = η	$A =$ vol. of 100 g. system	$A' =$ vol. of 100 g. system	Volume occupied by 1 g. dissolved substance $= \frac{A'}{A} = \frac{v}{c}$	Mean value of $\frac{v}{c}$
					vol. disperse phase (= v) $= \left(\frac{\eta}{\eta - 1} \right)^3$	weight of dissolved substance (= c) $= \frac{100}{\delta \times c}$		
12.95	$10^{-8.1}$	2.2	1.0582	21.69	1.152	7.298	6.335	6.51
10.81	"	1.8	1.0479	10.86	1.336	8.828	6.608	
9.84	"	1.7	1.0435	8.18	1.479	9.738	6.584	
6.60	"	1.1	1.0290	3.487	2.757	14.72	5.339	
3.27	"	0.5	1.0140	1.710	13.96	30.13	2.158	
5.68	$10^{-7.5}$	—	1.0164	12.17	1.294	17.32	13.39	6.57
"	$10^{-9.3}$	—	"	6.84	1.608	17.32	10.77	
"	$10^{-10.0}$	—	"	3.62	2.638	17.32	6.57	

TABLE VIII.

Volume occupied by the euglobulin phase in salt solution at 25° C.

(See Table III, Exp. II.)

Concentration of protein, % = c	Density of the system = δ	Coefficient of viscosity = η	$A =$ vol. of 100 g. of system	$A' =$ vol. of 100 g. of system	Volume occupied by 1 g. dissolved substance $= \frac{A'}{A} = \frac{v}{c}$	Mean value of $\frac{v}{c}$
			vol. of disperse phase (= v) $= \left(\frac{\eta}{\eta - 1} \right)^3$	wt. of dissolved substance (= c) $= \frac{100}{\delta \times c}$		
13.20	1.0650	29.56	1.109	7.114	6.415	6.51
12.32	1.0626	20.56	1.162	7.640	6.574	
10.57	1.0574	10.87	1.336	8.948	6.698	
8.86	1.0522	6.23	1.690	10.73	6.346	
6.58	1.0451	3.32	2.930	14.54	4.964	
1.36	1.0298	1.19	245.5	71.40	0.291	

TABLE IX.

*Volume occupied by the pseudoglobulin phase in solution at 25° C. (dialysed).
(See Table V.)*

Concentration of protein, % = c	Density of the system = δ	Coefficient of viscosity = η	$A =$ Total vol. of system vol. of disperse phase (= v) $= \left(\frac{\eta}{\eta - 1} \right)^3$	$A' =$ Total vol. of system wt. of dissolved substance (= c) $= \frac{100}{\delta \times c}$	Volume occupied by 1 g. dissolved substance $= \frac{A'}{A} = \frac{v}{c}$	Mean value of $\frac{v}{c}$
20.37	1.0619	38.79	1.082	4.623	4.272	4.50
18.45	1.0558	23.31	1.141	5.133	4.499	
16.43	1.0490	13.74	1.254	5.803	4.627	
14.06	1.0425	8.05	1.488	6.822	4.585	
11.82	1.0341	4.70	2.050	8.180	3.990	
10.04	1.0289	3.43	2.813	9.682	3.441	
8.32	1.0239	2.59	4.322	11.74	2.716	
5.95	1.0169	1.85	10.31	16.53	1.603	
3.61	1.0102	1.40	42.86	27.42	0.640	

TABLE X.

*Volume occupied by the serum albumin phase in solution at 25° C.
[See Chick and Lubrzynska, 1914, p. 65.]*

Concentration of protein, % = c	Density of the system = δ	Coefficient of viscosity = η	$A =$ vol. of 100 g. of system vol. of disperse phase (= v) $= \left(\frac{\eta}{\eta - 1} \right)^3$	$A' =$ vol. of 100 g. of system wt. of dissolved substance (= c) $= \frac{100}{\delta \times c}$	Volume occupied by 1 g. dissolved substance $= \frac{A'}{A} = \frac{v}{c}$	Mean value of $\frac{v}{c}$
20.65	1.0593	7.538	1.534	4.572	2.980	2.81
19.24	1.0555	5.875	1.751	4.924	2.838	
17.85	1.0513	4.763	2.028	5.329	2.628	
14.54	1.0413	3.025	3.332	6.605	1.982	
10.45	1.0296	1.952	8.618	9.295	1.078	
5.19	1.0153	1.316	72.20	18.98	0.263	
2.59	1.0075	1.128	684.0	38.32	0.056	

In all cases, with the exception of the experiment set forth in Table VII *b*, which will be discussed separately later on, the value $\frac{v}{c}$ remained constant when the disperse phase occupied more than one-half the total volume. It was greatest in the case of englobulin, one gram of which, in salt solution, was calculated to have a volume of 6.5 cc. while each gram of dissolved serum albumin was found only to occupy 2.81 cc., pseudoglobulin being intermediate between the two ($\frac{v}{c} = 4.52$). The solution volume of these

TABLE XI.

Water taken up by various proteins in the formation of colloidal solution at 25° C.

Protein	Density of the protein in solution at 25° (H ₂ O at 25° = 1)	Solution volume (as calculated from the density of the system)	Volume occupied by 1 g. protein in solution, cc.	Water associated with 1 g. protein, when in solution, cc.
Serum albumin (crystallised)	1.38	0.72	2.81	2.09
Pseudoglobulin	1.39	0.72	4.50	3.78
Euglobulin (salt)	1.42	0.70	6.51	5.81
Sodium caseinogenate	1.43	0.70	9.33	8.63
Egg-albumin (crystallised)	1.36	0.73	2.30	1.57

proteins can be calculated from the density of their solutions and hence the actual amount of water associated with each gram of protein can be calculated. These values vary from 5.8 cc. in the case of euglobulin to 3.8 cc. and 2.1 cc. for pseudoglobulin and serum-albumin respectively. These figures are all collected in Table XI, those for caseinogen and egg-albumin being added for purposes of comparison.

In order to institute useful comparison with the other proteins in serum, solutions of euglobulin in presence of salt have alone been taken into account in the preceding paragraph, because, in normal serum, it is under those conditions that this protein exists. When euglobulin is dispersed by alkali alone, its viscosity shows very remarkable variations, as may be seen from the results of Exp. I in Table II, where the euglobulin was practically salt-free. Under these circumstances the degree of viscosity depends upon the amount of alkali employed, being comparatively high at first and rapidly falling with increasing concentration of alkali and hydroxyl-ions. Increase of the latter from near the neutral point to a concentration of about 10^{-4} N (hydrogen-ion concentration 10^{-10} N) was accompanied, in the case of a 5.68 % euglobulin dispersion, by a fall in the viscosity coefficient from 12.17 to 3.62, which latter number is near the figure obtained for salt-globulin of the same protein-concentration. This decrease in viscosity is accompanied by a visual change, the globulin mixture, which remains in the form of a precipitate at concentrations of hydrogen-ions from about 10^{-5} to 10^{-6} N, changing to a thick, opalescent fluid at the neutral point (conc. $H^+ = 10^{-7}$ N) and to a thin, clear liquid at a concentration of hydrogen-ions equal to about 10^{-10} N. The observed change in size of the protein particles would, according to the theory of Hatschek, be unaccompanied by any change in viscosity unless there were a concomitant change in phase-ratio. In other

words, we must suppose that, in the more alkaline solution, the globulin phase contains less water and that the amount appropriated by the protein steadily increases as the iso-electric point is approached, with a consequent great increase in the volume of the disperse phase. In Table VII *b* the calculated value of $\frac{v}{c}$, the volume occupied by one gram protein in solutions of varying alkalinity, is seen to vary from 13.4 at the neutral point to 6.6 in the most alkaline solution employed. The latter figure, which is practically the same as that obtained for salt-globulin, is probably too low, for, in this case, the volume of the disperse phase, as ascertained by Hatschek's formula, is less than one-half the total volume and the calculation is therefore not strictly permissible.

In the presence of a small concentration of salt, the water appropriated by the globulin phase appears to be nearly independent of the reaction, the calculated value of $\frac{v}{c}$ being the same in the case of the two experiments in Table I and Table III, when the hydrogen-ion concentration was varied from 10^{-8} N to 10^{-6} N (see Tables VII *a* and VIII respectively).

The fact that the addition of alkali to a protein, with the consequent formation of protein salt and increase of protein ions, should be accompanied by a *lowering* of viscosity, and the conception that this is due to a less degree of association of the protein with water is opposed to the results of similar investigations with other proteins. Lacqueur and Sackur [1903] showed that increase in alkalinity led to an *increase* in viscosity in case of sodium caseinogenate. Pauli and Handovsky [1909 and 1910], working with the proteins of ox-serum, have shown that an addition of either acid or alkali leads to a preliminary rise in viscosity, followed by a fall as the concentration of either acid or alkali is further increased. Pauli and Falek [1912] have demonstrated the same phenomenon in case of gelatin solutions, and the work of Spiro [1904], Fischer [1910] and Chiari [1911] has shown that the water imbibed by gelatin increases rapidly as the reaction is made either more acid or more alkaline than the iso-electric point.

The general influence of salts¹ in lowering viscosity of protein solutions (and imbibition of water by gelatin), presumably due to their water-withdrawing capacity, was also demonstrated by the above observers and has been confirmed in the present work. The effect in the case of euglobulin is of great importance and has been fully discussed above; it is also well marked in the case of pseudoglobulin.

¹ What is possibly an analogous phenomenon with alcohol has been demonstrated by Brailsford Robertson [1912]. He found that the viscosity of sodium caseinogenate solutions (about 3 %) was lowered when the proportion of alcohol present was raised from 50 % to 75 %.

Influence of temperature. In the light of the theory developed above, the very marked influence of temperature upon the viscosity of protein solutions (see Table IV above, and Tables II, V and VII, Chick and Lubrzyńska [1914]) may be explained by a gradual loss of water from the protein phase as temperature is raised. It is usual, with most substances of crystalline nature, to find less water needed for the solution of each gram of the solute at a higher temperature, and the same property may also be assumed for substances of a colloidal nature. On such an assumption, the greater the initial appropriation of water, either due to the nature of the colloid or to its high concentration, the greater will be the loss of water from the disperse phase on rise of temperature and the diminution in its volume; in consequence the greater will be the fall in viscosity.

The experimental facts are in accord with this conception. With solutions of egg-albumin, where a comparatively small amount of water is assumed to enter into the composition of the disperse phase, the effect of temperature upon the viscosity coefficient is found to be negligible except in the case of very concentrated solutions (20% and upwards). On the other hand, comparatively dilute solutions of euglobulin, the most "hydrophile" of the proteins studied, show very marked changes in viscosity with alteration of temperature (see Table IV). In the following manner, I have attempted to estimate the volume occupied by one gram of (salt) euglobulin in solution at various temperatures. From curve *c*, Fig. 1, it is possible to determine the concentration of solutions which, at 25°, would possess the same viscosity coefficients as those determined for the 10.81% solution at the various temperatures (see the 4th column, Table IV). In the case of these solutions, at 25°, the concentration (in %) multiplied by 6.51, gives the volume (in cc.) occupied by the disperse phase. The same values for the volume of the disperse phase will apply to the 10.81% solution at the temperatures where the viscosity corresponds, if changes in viscosity due to alteration of temperature are assumed to be the result only of changes in phase-ratio (and if the differences in density of the solutions, caused by differences in temperature and concentration, are disregarded). In this way, the volume of one gram of euglobulin, in salt solution, at 2.1° has been estimated at 8.3 cc. and that at 43° at 5.3 cc.; at 25° the volume has already been calculated as 6.51 cc.

APPLICATION OF THE ABOVE RESULTS TO THE SALTING OUT OF PROTEINS.

The results of the preceding experiments, and the conceptions derived from them, throw a very interesting and illuminating light upon the phenomena displayed in the precipitation of proteins by neutral salts. They explain very clearly the influence of increased protein concentration, both in raising the proportion of protein thrown out by a given concentration of salt, and in lowering the limit of salt concentration at which precipitation occurs.

The values obtained, by the use of Hatschek's formula, for the amount of water presumably associated with unit weight of the various serum proteins in formation of colloidal solutions, yield a satisfactory explanation of the well-known precipitation limits of these proteins. The euglobulin, needing most water for its colloidal solution, is first driven out as water is withdrawn during any salting-out process; a higher concentration of salt is necessary to throw down the pseudoglobulin; while the albumin, which appropriates comparatively little water, is the last to be precipitated.

The current notion that albumin is less readily precipitated than the other proteins of serum, because it has a "greater affinity" for water, is thus seen to be erroneous. It is probably more consistent with the truth to affirm that the albumin, needing the exclusive use of less water for its own solution, is less liable to suffer precipitation when competition occurs.

SUMMARY.

1. The viscosity of solutions of pseudoglobulin and euglobulin from horse serum has been investigated as regards the influence of:

- (a) Concentration of protein.
- (b) Temperature.
- (c) Salt-content.
- (d) Hydrogen-ion concentration.

The results of similar experiments with serum albumin and whole serum, published previously by Chick and Lubrzenska [1914], are included in the general survey of the results.

2. In all cases, increase in protein concentration is accompanied by a disproportionately great increase in the viscosity of the solution. The effect is greatest in case of euglobulin, solutions of which exhibit a high viscosity at a comparatively low protein content. It is least in case of serum albumin, which, for strengths of protein under about 10%, behaves almost as a

crystalloid. Pseudoglobulin is intermediate between the other two proteins in this respect.

3. The viscosity of euglobulin solutions is dependent upon the manner in which solution (dispersion) of the protein is obtained. In case of "alkali globulin," where no salt is present, the viscosity of the solution depends upon the degree of alkalinity, falling rapidly with decrease in concentration of hydrogen-ions and distance from the iso-electric point. The viscosity of solutions of "salt-globulin" (NaCl) is considerably lower than that of alkali-globulin of equal protein and hydroxyl-ion content; in this case the viscosity is largely independent of the concentration both of salt (if above a small minimum, 0.5 to 1.0 %) and hydroxyl-ions. Euglobulin in serum is in the condition of salt-globulin.

4. The presence of a salt ($(\text{NH}_4)_2\text{SO}_4$) lowers the viscosity of pseudoglobulin solutions; it is without influence on the viscosity of solutions of albumin (egg) in concentrations up to 7 %.

5. The viscosity of protein solutions is decreased with rise of temperature frequently to a degree far in excess of that displayed by water or solutions of crystalloids. The greater the viscosity of the solution, the greater is the temperature effect, which is thus much enhanced in solutions of high protein concentration and most marked in case of euglobulin.

6. An interpretation of the above results is found in the two-phase nature of the systems studied. By means of Hatschek's formula, which gives an expression for phase-ratio in terms of viscosity, it is possible to calculate the relative volumes of protein- and water-phase in the more concentrated solutions employed. Hence values can be obtained for the volume occupied by one gram of the various proteins when in solution. These were found to show a satisfactory constancy and to be 2.8 cc., 4.5 cc. and 6.5 cc. for serum albumin, pseudoglobulin and salt-euglobulin respectively, at 25°. (The value obtained for alkali-euglobulin varied from 13.9 cc. to 6.6 cc. according to the alkalinity of the solution.) The solution volume of these proteins, reckoned from the density of their solutions, is equal to 0.7; the amount of water taken up by one gram of the protein at 25° would therefore be 2.1, 3.8 and 5.8 cc. respectively.

7. The conclusions given in no. 6 afford a satisfactory explanation of (a) the influence of protein concentration upon both the amount of protein precipitated, and the limit of salt concentration required to commence precipitation, in case of a pure protein; and (b) the relative order in which the above three proteins are precipitated from a mixture with increasing concentration of salt.

8. Hatschek's theory explains the observed disproportionate increase in viscosity of protein solutions with increase in concentration of the protein, the volume of the disperse phase being increased at the expense of the continuous phase. The relative magnitude of the phenomenon in case of the three proteins investigated is also interpreted.

9. The influence of temperature upon the viscosity of protein solutions would be explained by assuming a less degree of hydration of the colloid at higher temperature (an analogous phenomenon is encountered with crystalloids). A calculation has been made of the volume occupied by one gram of the protein at various temperatures in case of euglobulin. These volumes vary from 8.3 cc. at 2° to 5.3 cc. at 41°.

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VII. THE VISCOSITY OF SOME PROTEIN SOLUTIONS.

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(2 figures.)

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In the following work the same general methods were employed as in that already published by Martin and Chick [1912] upon the viscosity of solutions of caseinogen.

In the present instance an investigation was made of the influence of (1) concentration of protein, and (2) temperature, upon the viscosity of solutions of pure crystallised egg- and (horse) serum-albumin. For purposes of comparison, a few experiments were also made with whole serum, in some cases concentrated in vacuo in order to yield material of high protein content. The viscosity, as in the previous work, was determined by measuring the time of flow in an Ostwald viscosimeter, the figures obtained being relative to the time taken under similar conditions by pure water, which is expressed by unity. The concentrations throughout are expressed as grams per 100 grams of solution.

EGG-ALBUMIN.

The crystals of egg-albumin were obtained from egg-white by the method of Hopkins and Pinkus [1898]. The material was twice recrystallised, and then dialysed for two or three weeks against distilled water, till it contained an insignificant trace of ammonium sulphate. As considerable dilution took place during dialysis, it was necessary to concentrate the material in order to obtain the high concentrations necessary for the experiments. This was done by allowing the solution to evaporate at room temperature in vacuo over sulphuric acid, material being thus obtained which contained 28% by weight of egg-albumin. The protein-content in this and other cases was determined by boiling a weighed quantity of the solution, after dilution and acidification, and weighing the coagulum on a weighed filter, after drying at 110°.

TABLE I.

Influence of concentration of the protein upon the viscosity of solutions of pure egg-albumin (crystallised).

Temperature 25.2° C. Time of flow in viscosimeter for water 47.8 seconds.

Concentration of protein, %	Mean time of flow in viscosimeter, secs.	Density of the solution (H ₂ O at 25° C. = 1)	Coefficient of viscosity (H ₂ O = 1)
28.15	441.8	1.0805	9.99
26.83	367.4	1.0775	8.30
24.33	257.2	1.0693	5.81
20.12	159.2	1.0566	3.60
14.53	97.9	1.0402	2.21
8.877	69.4	1.0242	1.57
3.016	53.8	1.0083	1.22

Influence of concentration of Protein. In Table I are given the details of an experiment showing the influence of concentration upon the viscosity coefficient in the case of this protein. The same results are shown graphically in curve (a) of Fig. 1. Solutions containing different proportions by weight of protein were obtained by dilution of the concentrated material described above. In order to apply the necessary correction in calculating the coefficient of viscosity, the density of the solutions was directly determined by weighing a known volume in a pycnometer.

In the case of the weaker solutions, the viscosity remained low, increasing but slightly with increasing concentration of protein and only reaching a value equal to twice that of distilled water at a concentration of protein equal to about 13%. This is well seen in the curve in Fig. 1, where, up to a concentration of about 9%, the slope is very slight, in fact the relation between viscosity and concentration of protein approximates to that obtaining in solutions of non-colloidal material, and might satisfactorily be expressed by a straight line. At higher concentrations the curvature becomes increasingly greater until, at a concentration of 28% protein, the viscosity reaches the figure of nearly 10.

The curve expressing the relation between protein concentration and viscosity in case of solutions of egg-albumin is of the same general type as that obtained for caseinogen [Chick and Martin 1912]; in the case of the latter, however, a comparatively low concentration (7 to 8%) produced a viscosity equal to the value obtained with 28% egg-albumin. According to the hypothesis of Hatschek [1910, 1911, 1912] the difference between these two proteins in regard to their viscosity should be attributed to the fact that in a solution of egg-albumin much less water is appropriated

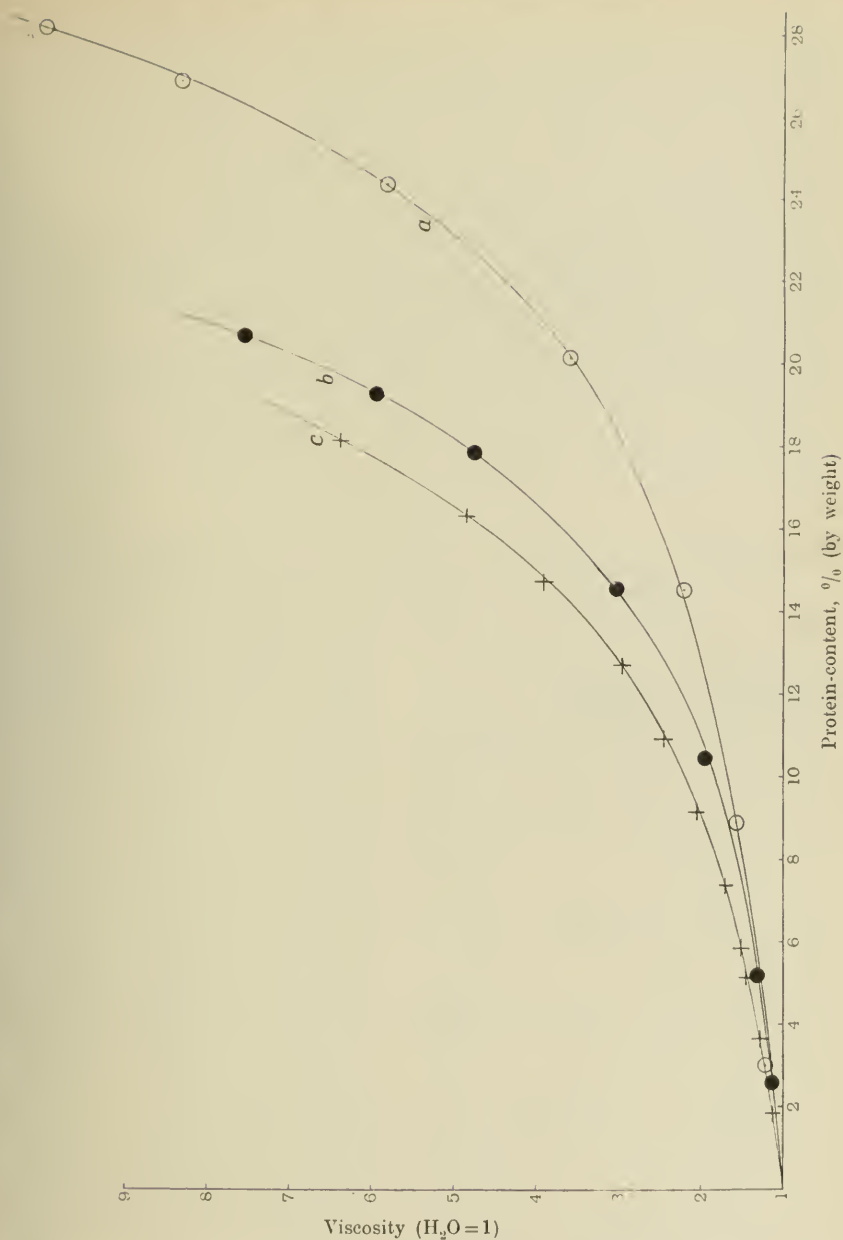


Fig. 1. Influence of protein-concentration upon the viscosity of solutions of various proteins.

- Curve (a) —○— = pure egg-albumin.
 .. (b) —●— = pure serum-albumin (horse).
 .. (c) —+— = whole serum, at protein-concentrations above and below the normal; original serum contained 7.3% protein.

by the protein phase than is the case with caseinogen. Consequently, in the former case, much higher concentration of protein is necessary to yield the high viscosity characteristic of the condition in which the aggregates of the disperse phase are approaching contact with one another. In a later communication it is proposed to discuss this theory as regards both egg-albumin and other proteins in greater detail.

Influence of Temperature. The variation of temperature was necessarily limited, the maximum range being from 0° to about 40°. Higher temperatures could not be employed without danger of the protein becoming "denatured."

In order to make the necessary comparison with the time of flow taken by water, experiments were made over the same range of temperature with an equal total volume of distilled water in each of the two viscosimeters employed in Exp. 1 and Exps. 2, 3 and 4 respectively. Smoothed curves

TABLE II.

Influence of temperature upon the viscosity of solutions of pure egg-albumin (crystallised) of varying concentration.

Exp. No.	Protein content, %	Density at 25° C. (H ₂ O at 25° C. = 1)	Temperature, °C.	Mean time of flow in viscosimeter, seconds		Coefficient of viscosity (H ₂ O at the same temperature = 1)
				Albumin solution	Distilled water (from curve)	
1	7.04	1.0192	2.8	136.8	101.8	1.37
			8.3	115.0	86.0	1.36
			15.2	95.0	70.3	1.38
			25.0	74.2	55.5	1.36
			32.3	63.0	47.9	1.34
			42.1	52.1	40.5	1.31
2	14.6	1.0404	2.8	179.2	86.2	2.16
			8.6	147.4	72.1	2.13
			14.7	123.4	61.5	2.09
			15.1	123.0	61.0	2.10
			25.0	94.0	48.0	2.04
			33.1	79.5	41.4	2.00
3	20.1	1.0566	0	368.3	96.4	4.04
			8.0	269.7	73.3	3.89
			17.0	202.3	58.0	3.68
			25.4	161.4	47.6	3.58
			33.0	132.1	41.4	3.37
			41.6	109.5	35.4	3.27
4	28.15	1.0805	0.6	1170.2	93.8	13.48
			7.8	828.6	73.8	12.13
			15.6	614.1	60.1	11.04
			25.4	440.9	47.6	10.01
			33.9	314.9	40.8	9.13
			41.9	283.7	35.1	8.73

were drawn through the experimental points and from these the time of flow corresponding to any intermediate temperature could be read off. The values given in the 5th column of Table II were obtained in this way and, with the help of these, the coefficients of viscosity in the 7th column were calculated.

In case of the weaker solutions (from 7% up to 20%) the influence of temperature upon the viscosity was comparatively trivial, that is to say, the phenomenon was of the same order as that obtaining in the case of distilled water. A 7% solution of egg-albumin, with viscosity of 1.3, behaved on heating as pure water containing a crystalloid in solution. Even with a 20% solution, the viscosity relative to water was only about 20% greater

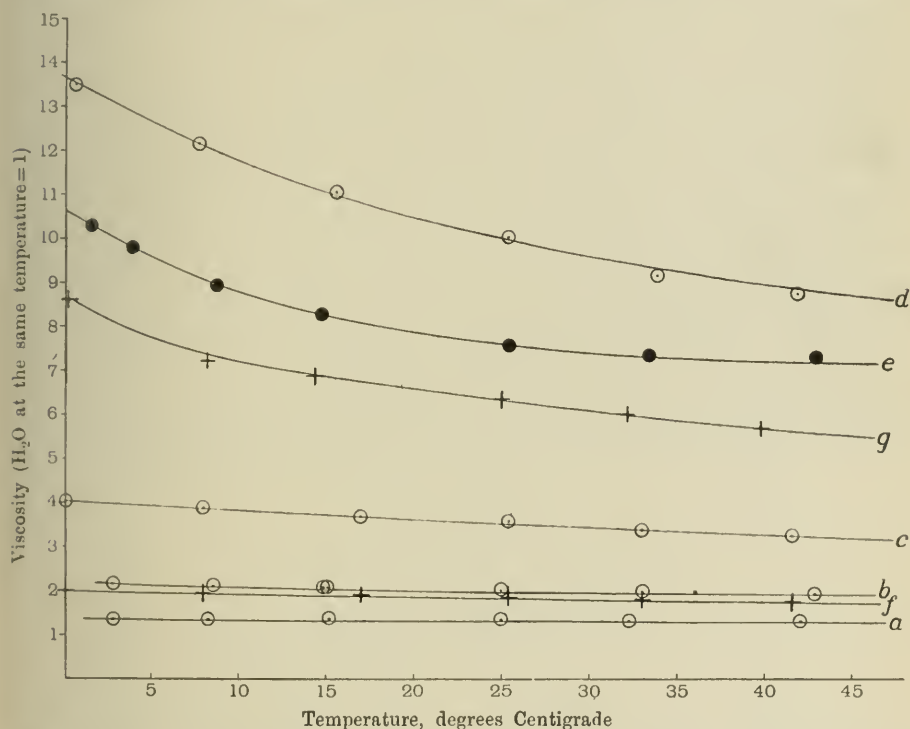


Fig. 2. Influence of temperature upon the viscosity of solutions of various proteins with differing protein-content.

- = egg-albumin: curve a, protein content = 7.0%.
- " b, " " = 14.6 "
- " c, " " = 20.1 "
- " d, " " = 28.1 "
- = serum protein: curve e, " " = 20.6 "
- +— = whole serum: curve f, " " = 7.0 "
- " g, " " = 18.1 "

at 0° than at 41·6°, and not until the concentration of albumin reached the high figure of 28%, was temperature found to have any specially marked influence.

These facts are well expressed in the four curves *a*, *b*, *c* and *d* in Fig. 2, where viscosity (relative to water at the same temperature) is plotted as ordinate against temperature as abscissa.

Influence of Ammonium Sulphate upon Viscosity of Crystallised Egg-albumin. It has been shown [Chick and Martin 1913, 2; Spiro 1904] that "precipitation" of egg-albumin by ammonium sulphate is a phase-separation and that a definite proportion of both salt and water is associated with the egg-albumin in the "precipitate." Arguing from analogy, it is exceedingly probable that crystallisation of proteins from strong solutions of ammonium sulphate is of a similar character.

In order to ascertain whether such hypothetical association with ammonium sulphate had any influence on the viscosity of solutions, strictly comparable experiments were made, both before and after dialysis. Dabrowski [1912], on the basis of a comparison between the different rates of diffusion obtained with the two kinds of material, came to the conclusion that aggregates formed in solution by undialysed crystals of egg-albumin were much smaller, nearly one-sixth the size of those of dialysed albumin. It therefore seemed possible that such a fundamental difference in the type of solution might also be expressed by some significant change in viscosity.

The results obtained were, however, entirely negative (see Table III). Solutions were made of exactly similar protein content, 6·98%, in the one

TABLE III.

Viscosity of crystallised egg-albumin.

(a) Before, and (b) after dialysis.

Exp.	Protein content, %	Ammonium sulphate, %	Temperature, °C.	Mean time of flow in viscosimeter, seconds		Coefficient of viscosity ((NH ₄) ₂ SO ₄ solution in exp. (a) and H ₂ O in exp. (b) = 1)
				Albumin solution	3·51% (NH ₄) ₂ SO ₄ solution in exp. (a) and H ₂ O in exp. (b)	
(a)	6·98 ¹	3·51	0·6	145·9	109·9	1·35
	"	"	25·5	75·9	58·8	1·31
	"	"	45·1	51·4	39·8	1·32
(b)	6·98 ¹	—	1·0	145·4	109·0	1·36
	"	—	25·4	74·5	57·0	1·33
	"	—	45·1	49·2	38·3	1·31

¹ Density of the solution taken as 1·019 for the purpose of calculating viscosity coefficient.

case from undialysed crystals, with their ammonium sulphate (= 3.51 %), and in the other from dialysed material. In the former case viscosity was determined relative to pure water and in the latter case in relation to a 3.51 % solution of ammonium sulphate. Comparison was instituted at three different temperatures. Temperature was not found to have any unusual influence on viscosity, nor was any difference traced between the two types of solution. At the same time it must be admitted that the concentration of protein employed (7 %) was rather low, and it would be well to repeat the experiment with stronger solutions.

SERUM-ALBUMIN (HORSE).

A series of experiments similar to those with egg-albumin were also carried out with pure serum-albumin. The protein in this case was also crystallised in presence of ammonium sulphate according to the method of Hopkins and Pinkus [1898] and, after recrystallisation, dialysed against distilled water in presence of toluene for some weeks. A solution was obtained, containing 20.65 % protein and traces only of ammonium sulphate.

Influence of Concentration of Protein. The density of serum-albumin solutions has been shown to bear a linear relation to the concentration of protein [Chick and Martin 1913, 1]. In order, therefore, to apply the appropriate correction in calculating the coefficient of viscosity it was only necessary to make direct determinations in a few cases. The straight line could then be drawn expressing the relation of density to protein content and the other values of density required be obtained by interpolation; such values are marked with an asterisk in Table IV.

TABLE IV.

Influence of concentration of the protein upon the viscosity of pure serum-albumin (crystallised).

Temperature 25.4° C. Time of flow in viscosimeter for water 55 seconds.

Concentration of protein, %	Mean time of flow in viscosi- meter, secs.	Density of the solution (H ₂ O at 25° C. = 1)	Coefficient of viscosity, H ₂ O = 1
20.65	391.4	1.0593	7.54
19.24	310.2	1.0549*	5.95
17.85	249.2	1.0509*	4.76
14.54	159.8	1.0412	3.02
10.45	104.3	1.0296	1.95
5.19	71.3	1.0153	1.32
2.59	61.6	1.0075	1.13

* Interpolated values.

The experiments showing the influence of protein content on viscosity are detailed in Table IV and graphically expressed in Fig. 1, curve (*b*), where viscosity as ordinate is plotted against concentration of protein as abscissa.

The results show great similarity with those obtained for egg-albumin. In Fig. 1, curves (*a*) and (*b*) run closely together at first. As the concentration of protein increases, however, it is seen that serum-albumin has a much higher viscosity than egg-albumin.

Influence of Temperature. The influence of temperature was investigated in one experiment only, of which details are given in Table V, and graphically expressed in curve (*e*) Fig. 2. This experiment was made with the most concentrated solution available, containing 20.1 % protein by weight. The viscosity relative to water was not only much greater than that of egg-albumin of similar strength (see Exp. 3, Table II), but showed a much greater change with alteration of temperature, viz. from 7.3 at 43° to 10.3 at 1.6° (compare Table V with Exp. 3, Table II).

TABLE V.

Influence of temperature upon the viscosity of pure serum-albumin (crystallised).

Protein-content = 20.65 %. Density = 1.0593 (at 25° C.).

Temperature, °C.	Mean time of flow in viscosimeter, seconds		Coefficient of viscosity (H ₂ O at the same temperature = 1)
	Albumin solution	Distilled water (from curve)	
1.6	1026.0	105.6	10.29
3.95	906.2	98.2	9.77
8.8	713.2	84.7	8.92
14.8	553.8	71.0	8.26
25.4	391.4	54.8	7.56
33.4	325.8	47.0	7.34
43.0	275.1	40.0	7.28

WHOLE SERUM (HORSE).

The sample of horse-serum selected for these experiments contained 7 % total protein. In order to obtain solutions of protein-content comparable to those employed above, some concentration of the serum proteins was necessary. By placing in shallow dishes for 48 hours in vacuo at room temperature material was readily prepared, which contained 18 % protein. The constituents of the serum did not appear to have been affected by the process, for, after diluting the concentrated material to obtain a solution whose protein-content was equal to that of the original serum, the viscosity was also found to be the same as that previously determined (see Table VI).

Influence of Concentration of Protein and of Temperature. The proportion of salt contained in serum was found to have an insignificant influence upon both the density and the viscosity of the system; hence the values of the coefficient of viscosity were calculated in relation to pure water as before. In Table VI are given the results of a series of experiments in which the protein-content varied from 1.8% to 18% by weight.

In obtaining values for the density of the various solutions, the same method was adopted as in the case of serum-albumin; after a direct determination had been made in case of one or two solutions, a curve was constructed from which intermediate values could be obtained. Such interpolated values are marked with an asterisk in Table VI.

TABLE VI.

Viscosity of horse-serum at concentrations above and below the normal; the normal serum contained 7.3% protein.

Temperature 25° C. Time of flow in viscosimeter for water 55.5 seconds.

Concentration of protein, %	Time taken for flow in viscosimeter, secs.	Density of the solution (H ₂ O at 25° C. = 1)	Coefficient of viscosity (H ₂ O = 1)
1.836	62.4	1.0069*	1.13
3.665	70.3	1.0143	1.28
5.134	79.4	1.0193*	1.46
5.84	82.3	1.0220*	1.52
7.31 ¹	92.8	1.0276	1.72
7.33 ²	92.5	1.0276	1.71
9.13	110.2	1.0344*	2.05
10.89	131.3	1.0405	2.46
12.67	156.6	1.0477*	2.96
14.71	206.2	1.0553*	3.92
16.27	253.6	1.0617	4.85
18.10	331.5	1.0679*	6.38

¹ Normal serum.

² Prepared by dilution from concentrated serum.

* Interpolated and extrapolated values.

From Table VI, and more readily from curve (c) Fig. 1, it is seen that for low concentrations of total protein the viscosity of whole serum also remained low, varying from about 1.10 to 1.7 as the concentration of total protein was raised from 1.80% to that of the normal serum (7.3%). As the serum was concentrated, however, the viscosity increased rapidly with increasing concentration of total protein, and when it possessed a little more than twice the normal protein-content the viscosity relative to water reached the value of 5.0.

In comparison with serum- or egg-albumin the total proteins of serum yield much higher viscosity when in solution. For example, while 18% solutions of serum- and egg-albumin gave coefficients of viscosity equal to 4.9 and 2.8 respectively (see curves (b) and (a), Fig. 1), concentrated whole serum containing the same proportion of total protein had a viscosity of 6.38.

The influence of temperature upon viscosity was also greater with the proteins of whole serum. With the concentrated material, containing 18% protein, the coefficient of viscosity fell 34% (from 8.63 to 5.7) with increase in temperature of about 40° (see Table VII). A slightly greater rise of temperature in case of solutions containing 20% serum- and egg-albumin produced a decrease in viscosity coefficient of only about 29% and 19% respectively (see Tables V and II).

TABLE VII.

*Influence of temperature upon the viscosity of whole serum (horse),
(a) normal serum, (b) concentrated serum.*

Exp.	Protein content, %	Density at 25° C. (H ₂ O at 25° C.=1)	Temperature, °C.	Mean time of flow in viscosimeter, seconds		Coefficient of viscosity (H ₂ O at the same temperature = 1)
				Serum	Distilled water (from curve)	
(a)	7.68	1.0290	0	218.2	111.6	2.01
	"	"	8.0	164.9	86.7	1.96
	"	"	17.0	125.5	67.1	1.92
	"	"	25.4	99.6	54.9	1.87
	"	"	33.0	83.6	47.3	1.82
	"	"	41.6	70.4	40.9	1.77
(b)	18.1	1.0679	0.2	897.2	111.0	8.63
	"	"	8.2	581.5	86.0	7.22
	"	"	14.3	462.3	71.9	6.86
	"	"	25.0	328.9	55.4	6.34
	"	"	32.2	270.3	48.0	6.01
	"	"	39.8	224.6	42.1	5.70

It is evident that one or more of the remaining proteins in horse serum must, when in solution, possess a viscosity much higher than that of the serum-albumin, and the results of some preliminary experiments suggest that the small proportion of "euglobulin" contained in the serum exercises a disproportionate influence upon the viscosity of the whole.

In a second communication it is proposed to publish the results of experiments, similar to the above, carried out with purified samples of

"euglobulin" and "pseudoglobulin," prepared from horse serum, and to include a general discussion of the theoretical bearing of the whole series of data.

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XXXIX. THE PRECIPITATION OF EGG-ALBUMIN BY AMMONIUM SULPHATE. A CONTRIBUTION TO THE THEORY OF THE "SALTING-OUT" OF PROTEINS.

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INTRODUCTION.

The first systematic and quantitative researches upon the precipitation of proteins by salts in large amounts were made by Hofmeister and his pupils.

In the experiments of Kauder [1886] and Lewith [1888] with serum proteins, and Hofmeister [1888] with egg-protein, comparison was made of the precipitating power of a large series of electrolytes, as a result of which the latter were arranged in what is known as the Hofmeister series. Most of the experiments were made with sodium salts, among which sulphate, phosphate, acetate, citrate, tartrate, chromate, chloride, nitrate and chlorate formed a series arranged in descending power of precipitation; of the kations examined lithium was the most effective, and sodium, potassium, ammonium and magnesium came afterwards in order of decreasing efficiency.

Hofmeister [1889] came to the conclusion that the precipitation was caused by the electrolytes depriving the protein of the amount of water necessary to keep it in solution, and was confirmed in this view by the results of some experiments showing the influence of various salts in modifying the imbibition of water by gelatin [1891]; work in this direction was later extended by Pauli [1898] and Pauli and Rona [1902].

Spiro [1904] demonstrated that the precipitation from their solutions of caseinogen and gelatin by sodium sulphate was analogous to the "salting out" of alcohol recently studied in detail by de Bruyn [1900]. In neither case is the phenomenon one of simple precipitation, since, owing to the appropriation of water by the salt, separation into two phases occurs. Each phase contains all the constituents of the system and any alteration in one of

the three constituents leads to readjustment of the composition and relative volumes of the two phases. Spiro also pointed out that, since in the case of alcohol the effect of electrolytes is not attributable to the constituent ions, any influence of the latter in the salting out of proteins must be regarded as a subsidiary phenomenon.

Spiro's conception explains to some extent the divergent results obtained in the precipitation of proteins by the addition of neutral salts, when the whole conditions are not maintained constant.

The series of observations which we are about to record concern the "salting out" by ammonium sulphate of pure recrystallised egg-albumin. Our observations show that in this case also we have to deal with the separation of the original system (itself not homogeneous) into two distinct phases, and that the influence upon the volume of these phases of concentration of protein, salt and water in the system is, as Spiro found for caseinogen and gelatin, analogous to what occurs in alcohol, salt and water mixtures. In addition, however, we find that the charge carried by the protein particles is an important factor in the final equilibrium.

The results of the experiments will first be set forth and the proposed explanation discussed later.

PRECIPITATION OF PURE EGG-ALBUMIN BY AMMONIUM SULPHATE.

Material. The material employed was egg-albumin crystallised from egg-white in presence of ammonium sulphate according to the method of Hopkins and Pinkus [1898]. The albumin was recrystallised once or twice, separated from the mother liquor by pressing between filter paper, and finally dissolved in distilled water. A concentrated stock solution was thus obtained, the composition of which, as regards (1) protein, (2) ammonium sulphate, (3) water was accurately ascertained by analysis, and which, when diluted to a suitable degree, served for most of the following experiments.

Since the salt employed for "salting out" in these experiments was also ammonium sulphate, the small concentration of the latter always present in the original albumin solution presented no complication; an allowance was made for this amount in the calculations. In those cases where an electrolyte-free solution was required, the albumin solution was previously dialysed.

Egg-albumin prepared in this way we believe to be as homogeneous a protein as it is possible to obtain. Hopkins [1899-1900] came to the conclusion that egg-albumin, crystallised from faintly acid ammonium sulphate solutions by the above method, was a pure substance. The

rotatory power remained absolutely constant after repeated recrystallisations (p. 312) and the proportion of carbon, hydrogen, nitrogen and sulphur, as well as the ash, remained constant. His experiments were made with four different samples after three or four recrystallisations.

1. *Influence of concentration of salt upon the amount of protein precipitated.*

Mellanby [1907] made a quantitative study of the influence of concentration of ammonium sulphate on the precipitation of the proteins from horse serum, but, as far as we are aware, no experiments have as yet been made with a pure protein. In the present instance two sets of experiments were made, in both of which the concentration of protein was about 1 %. In the first the concentration of protein in the whole system was left constant = 1.11 % by weight, and the ratio salt to water was varied (Table I and Fig. 1). In the second set the ratio protein to water was kept constant and the precipitation studied by varying the amount of salt present; the concentration of protein in the whole system varied from 1.0 % to 0.93 % (by weight). (Table II and Fig. 2.)

TABLE I.

Precipitation of pure egg-albumin with ammonium sulphate; influence of concentration of salt.

Temperature, 20°.

Protein constant = 1.11 % by weight of total system.

Ratio salt/water varying.

Albumin g.	Water g.	Salt g.	G. albumin in 100 g. total system	G. salt in 100 g. total system	G. albumin in 100 g. filtrate
1.00	69.00	20.00	1.11	22.22	1.089
1.00	68.00	21.00	"	23.33	0.711
1.00	67.00	22.00	"	24.44	0.302
1.00	66.00	23.00	"	25.55	0.104
1.00	65.00	24.00	"	26.66	0.0315
0.50	32.35	12.15	"	27.00	trace
0.50	32.00	12.50	"	27.77	trace

The method of experiment was as follows. Mixtures were prepared by weighing into stoppered bottles the required amount of water and protein, and the necessary amount of ammonium sulphate was then added in large crystals and gently shaken. This prevented over-saturation with ammonium sulphate in the neighbourhood of the crystals, the large size of which

prevented a too rapid solution. The bottles were placed in a thermostat at 20°, for from 1 to 2 hours, the contents filtered and the protein estimated in the filtrate by weighing the coagulum formed on heating.

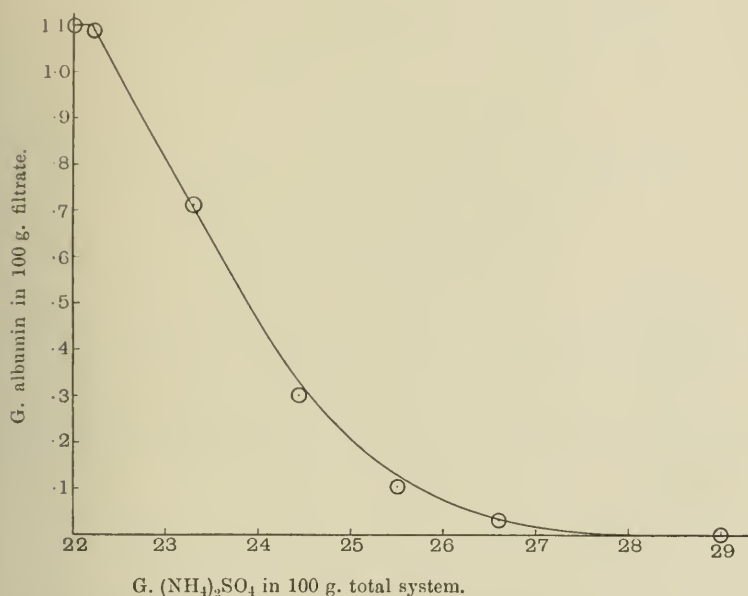


Fig. 1. Influence of concentration of the salt upon precipitation of egg-albumin by $(\text{NH}_4)_2\text{SO}_4$ at 20°, see Table I; protein constant = 1.11 % (by weight) of total system, ratio salt to water varying.

TABLE II.

*Precipitation of pure egg-albumin with ammonium sulphate;
influence of concentration of salt.*

Temperature, 20°.

Ratio protein/water constant = 1.3/100.

Albumin g.	Water g.	Salt g.	G. salt to 1.3 g. protein and 100 g. H ₂ O	G. albumin in 100 g. filtrate
1.30	100	29	29	0.998
1.3	100	29.7	29.7	no precipitation.
1.30	100	30	30	0.938
1.95	150	46.5	31	0.733
1.3	100	32	32	0.487
2.6	200	66	33	0.273
2.6	200	67	33.5	0.232
2.6	200	70.01	35.0	0.105
2.6	200	76	38.0	0.022

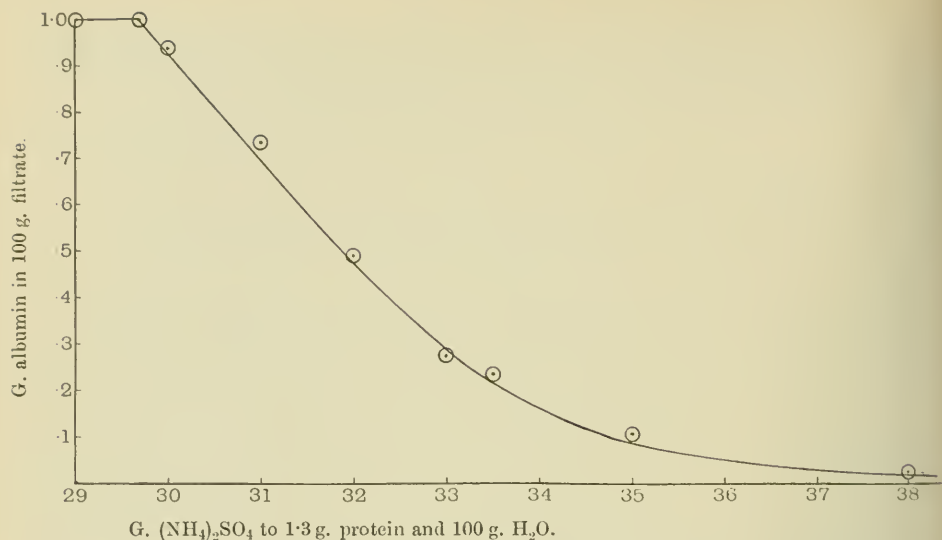


Fig. 2. Influence of concentration of the salt upon the precipitation of egg-albumin by $(\text{NH}_4)_2\text{SO}_4$ at 20° , see Table II; ratio protein to water constant = 1.3 to 100, amount of salt present varying.

The curves in Figs. 1 and 2 are both of the same general form, in both cases the percentages of albumin in the filtrate are plotted as ordinates and the abscissae are respectively percentage by weight of ammonium sulphate in the whole system and grams of ammonium sulphate present, which explains the fact that the curve is steeper in the former instance. The point of commencing precipitation which is very sharply marked is at about the same concentration of salt in both cases, viz. 22.2 and 22.7 % ammonium sulphate respectively; the curve then descends steeply and approaches the base line asymptotically.

2. Influence of concentration of protein.

Kauder [1886] showed that serum albumin was more readily precipitated by ammonium sulphate if in more concentrated solution and determined the diminishing concentration of ammonium sulphate necessary to cause commencing precipitation in a series of solutions of increasing protein concentration. Hofmeister in 1888 published the results of similar experiments, using egg-white and potassium acetate and ammonium sulphate. Similar evidence has since been brought forward by other workers, e.g. Mellanby [1907], but in no case was a pure protein employed.

We have made an experiment with pure egg-albumin, estimating the

protein precipitated by a constant concentration of ammonium sulphate (ratio salt to water constant) when the amount of protein was varied. The results are given in Table III and graphically set forth in Fig. 3, where the proportion of the protein separated is plotted as ordinate against the concentration (percentage by weight of whole system) of protein in the original mixture as abscissa. Not only is more protein separated from the more concentrated

TABLE III.

*Precipitation of pure egg-albumin with ammonium sulphate;
influence of concentration of protein.*

Ratio salt/water constant=31/100.

Concentration of protein varying.

Albumin g.	Water g.	Salt g.	G. albumin in 100g. total mixture	G. albumin to 31 g. salt and 100g. H ₂ O	G. albumin in 100g. filtrate	G. albumin precipitated from 100g. total mixture	Protein pptd. %
1.90	56.92	17.65	2.481	3.33	1.130	1.351	54.4
1.90	36.92	11.44	3.775	5.14	1.115	2.660	70.4
3.79	50.85	15.76	5.383	7.45	1.159	4.224	78.5
7.59	53.69	16.64	9.738	14.13	0.935	8.803	90.4
4.74	17.31	5.36	17.306	27.4	0.772	16.534	95.5

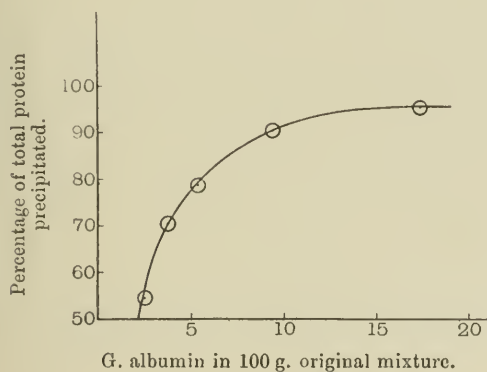


Fig. 3. Influence of concentration of protein upon the precipitation of egg-albumin by $(\text{NH}_4)_2\text{SO}_4$ at $20^\circ\text{C}.$, see Table III; ratio salt to water constant=31 to 100, amount of protein varying.

solution at a given concentration of salt, but a greater proportion is precipitated (see last column, Table III). The concentration of protein in the filtrate is not constant, but varies from 1.13 % to 0.77 % (col. 6, Table III), as the initial protein concentration is varied from 2.5 to 17.3 % (col. 4). This suggests that the precipitation is a phase separation, analogous to the case of "salting out" of alcohol with ammonium sulphate. As will be

seen later, when the results are given of determinations of the protein-salt-water content of filtrate and precipitate respectively, this was proved to be the case.

3. *Influence of hydrogen ion concentration.*

It is common experience, e.g. with serum, that the addition of a little acid enhances the amount of protein precipitated by the same concentration of ammonium sulphate, and that proteins not precipitated by saturation with sodium chloride are thrown down on acidification of the solution.

Mellanby [1907] called attention to the increased amount of precipitation of horse serum by neutral salts after addition of various acids, and gave some quantitative data, using sodium chloride. In the present investigation the influence of acidity was directly measured in a series of mixtures in which the concentration of protein and ammonium sulphate was maintained constant, and so chosen that precipitation had just begun in the control solution. The reaction of the solution was adjusted to various degrees of hydrogen ion concentration by the addition of small quantities of standard sulphuric acid.

Determinations of hydrogen ion concentration were made with the type of concentration cell described by Michaelis and Rona [1909], the contact fluid between the two cells being saturated potassium chloride solution. It is possible that the determinations of H^+ concentration are not very accurate owing to the high concentration of ammonium sulphate present; those in any one series are, however, perfectly comparable.

TABLE IV.

*Precipitation of pure egg-albumin with ammonium sulphate;
influence of hydrogen ion concentration.*

Experiment I at 18°.

G. ammonium sulphate in 100 cc. original mixture = 30.4.

G. protein in 100 cc. original mixture = 0.575.

G. protein present in 100 cc. filtrate	Hydrogen ion concentration in filtrate, in terms of normality
0.572	$10^{-5.27}$ (54×10^{-7})
0.391	$10^{-5.00}$ ($101 \times \text{,,}$)
0.097	$10^{-4.64}$ ($231 \times \text{,,}$)
0.062	$10^{-4.53}$ ($296 \times \text{,,}$)
0.048	$10^{-4.31}$ ($492 \times \text{,,}$)
0.035	$10^{-4.11}$ ($780 \times \text{,,}$)

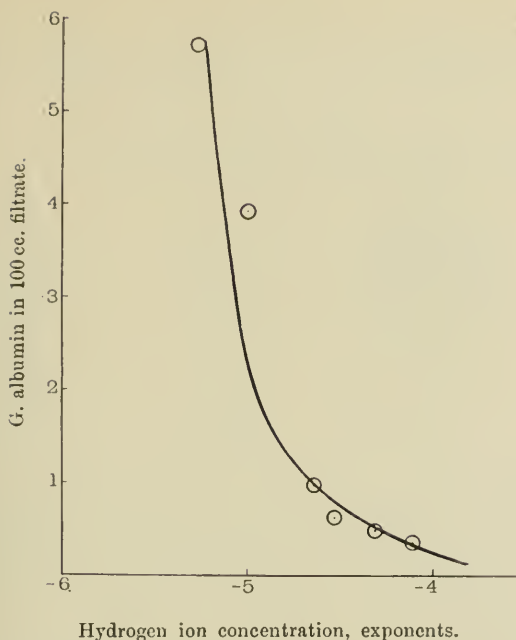


Fig. 4. Influence of hydrogen ion concentration upon the precipitation of egg-albumin by $(\text{NH}_4)_2\text{SO}_4$ at 18° (see Table IV).

Concentration of $(\text{NH}_4)_2\text{SO}_4$ in whole system, constant = 30.4 grams per 100 cc.

„ „ „ Protein „ „ „ = 0.575 „ „

The concentration of protein varied from 0.6 to 0.9% and that of ammonium sulphate from 30.4 to 28.6% (by volume) in the three different series of experiments set forth in Tables IV to VI. The mixtures were placed for two hours at 18° in order that equilibrium might be attained; they were then filtered and the concentration of protein and of hydrogen ions in the filtrate was determined.

The influence of hydrogen ion concentration was found to be very marked although the range through which it operates is not extensive. In one case (Exp. I, Table IV, protein concentration = 0.57%, ammonium sulphate = 30.4%) an increase in acidity from 54×10^{-7} normal (control) to 780×10^{-7} normal was enough to cause precipitation of nearly all the protein. In Exp. II, Table V (0.86% protein and 30.03% ammonium sulphate) and Exp. III, Table VI (0.9% protein and 28.6% ammonium sulphate) the effective range of hydrogen ion concentration was from 2.9 to 360×10^{-7} normal, and from 12 to 203×10^{-7} normal respectively.

TABLE V.

*Precipitation of pure egg-albumin with ammonium sulphate;
influence of hydrogen ion concentration.*

Experiment II at 18°.

G. ammonium sulphate in 100 g. original mixture = 30.03.

G. protein in 100 g. original mixture = 0.856.

No. of cc. N/10 H ₂ SO ₄ (or equivalent) added in total volume of 27 cc.	No. of cc. N/10 NH ₄ OH (or equivalent) added in total volume of 27 cc.	G. protein in 100 cc. filtrate	Hydrogen ion concentra- tion (filtrate), in terms of normality
—	1.0	0.864	10 ^{-6.54} (2.88 × 10 ⁻⁷)
—	0.5	0.809	10 ^{-6.01} (9.82 × „)
—	0.3	0.795	10 ^{-5.72} (19.0 × „)
—	—	0.514	10 ^{-5.40} (39.8 × „)
0.5	—	0.056	10 ^{-4.98} (104 × „)
1.0	—	0.01 (about)	10 ^{-4.45} (358 × „)

The last trace of protein present, however, does not appear to be precipitated by alteration of reaction alone, a slight trace in Exp. III being still left in solution at a hydrogen ion concentration of about 1/100 normal or 73000×10^{-7} normal.

The range of reaction where hydrogen ion concentration has its great effect is presumably just on the acid side of the iso-electric point (see below p. 392). From Exp. III it is seen that for ammonium sulphate change in hydrogen ion concentration at and on the alkaline side of the neutral point is without much influence (viz. from $H^+ = 12 \times 10^{-7}$ normal to 0.008×10^{-7} normal), see Table VI.

TABLE VI.

*Precipitation of pure egg-albumin with ammonium sulphate;
influence of reaction, hydrogen ion concentration.*

Experiment III at 18°.

G. (NH₄)₂SO₄ in 100 g. original mixture = 28.6.

G. protein „ „ „ „ = 0.910.

Cc. N/10 H ₂ SO ₄ (or equivalent) added in total volume of 25.4 cc.	Cc. N/10 (NH ₄)OH (or equivalent) added in total volume of 25.4 cc.	G. protein in 100 cc. filtrate	Hydrogen ion concentra- tion (filtrate) in terms of normality
—	3.6	No precipitation	10 ^{-9.11} (0.008 × 10 ⁻⁷)
—	2.0	0.899	10 ^{-7.23} (0.59 „)
—	1.0	0.908	10 ^{-6.56} (2.7 „)
—	—	0.899	10 ^{-6.08} (12.0 „)
0.25	—	0.533	10 ^{-5.45} (35 „)
0.5	—	0.174	10 ^{-5.01} (97.5 „)
1.0	—	0.063	10 ^{-4.63} (203 „)
5.0	—	trace	10 ^{-2.45} (35000 „)
10.0	—	slight trace	10 ^{-2.14} (73000 „)

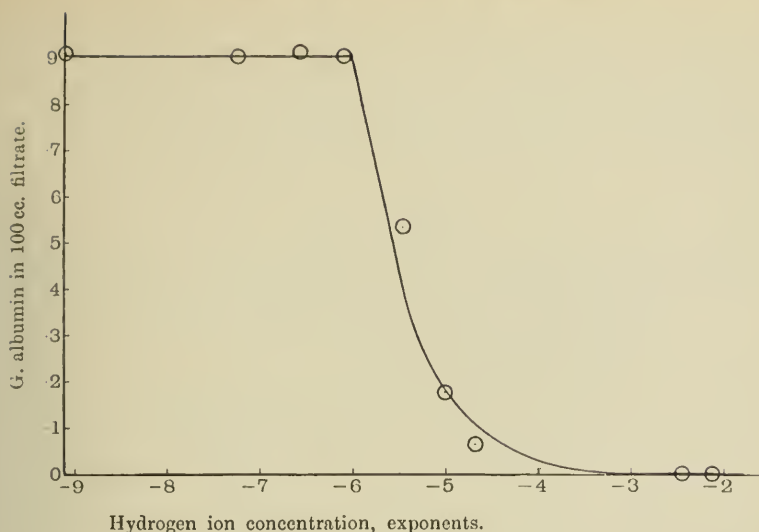


Fig. 5. Influence of hydrogen ion concentration upon the precipitation of egg-albumin by $(\text{NH}_4)_2\text{SO}_4$ at 18° (see Table VI).

Concentration of $(\text{NH}_4)_2\text{SO}_4$ in whole system, constant = 28.6 grams in 100 cc.

" " protein " " " = 0.910 " "

H^+ concentration expressed as exponents.

4. Influence of temperature.

Lewith [1888] in case of ox-serum proteins, showed that rise in temperature assisted the precipitation by ammonium sulphate and Hofmeister [1888] made the same observation with egg-white and various salts. Spiro [1904] stated the same to be true of crystalline serum albumin and ammonium sulphate.

We have confirmed the above observations for serum proteins¹ and pure egg-albumin if the reaction be alkaline, but in faintly acid solution (10^{-5} normal) we have found the reverse to be true above 9° .

In Table VII and Fig. 6 are set forth the results of an experiment with 0.85 % protein and 28 % ammonium sulphate. A series of exactly similar solutions were placed for from 1 to 2 hours in a thermostat at the required temperature, after which they were rapidly filtered and the protein estimated in the filtrate. From 0° to 9° the temperature coefficient of precipitation was positive, above 9° it remained negative to 50° ². This can be readily

¹ Mellanby [1907, p. 294], on the other hand, states that the temperature coefficient of serum-protein precipitation with $(\text{NH}_4)_2\text{SO}_4$ is negative between the temperatures of 0° and 40°C. and so small as to be unimportant.

² No denaturation occurred.

demonstrated if ammonium sulphate be added to an egg-albumin solution at 9° to the point of opalescence, and just short of the formation of a definite precipitate. If, then, the mixture be divided into three portions, of which one is placed at 0° and a second at 20° , a definite precipitation will occur at both temperatures, whereas the portion maintained at 9° will remain merely opalescent.

TABLE VII.

Influence of temperature upon the precipitation of pure crystalline egg-albumin with ammonium sulphate (28 %).

Protein content = 0.85 %.

Hydrogen ion concentration about 10^{-5} normal.

Temperature	Concentration of protein in filtrate, %
50	0.828
36	0.703
20	0.364
9	0.268
0	0.318

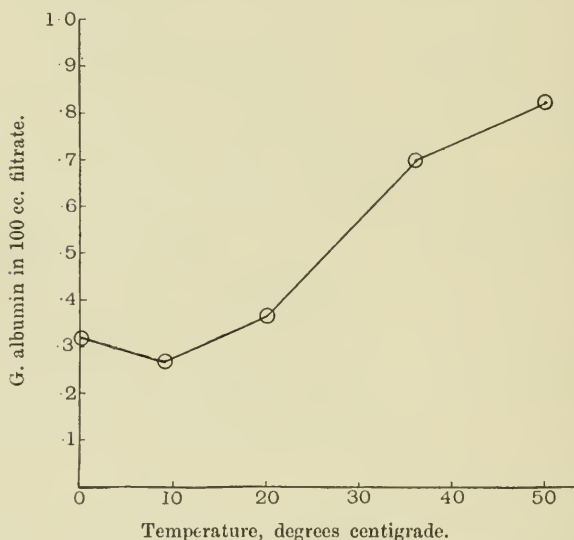


Fig. 6. Influence of temperature upon the precipitation of egg-albumin by $(\text{NH}_4)_2\text{SO}_4$, see Table VII.

Concentration of protein, salt, water and hydrogen ions in whole system constant.

INTERPRETATION OF RESULTS.

From the experiments detailed above, it is clear that the amount of protein precipitated from solution (volume of the protein-rich phase) is

dependent upon the amount of salt present, concentration of protein, concentration of hydrogen ions and temperature.

Spiro [1904] showed that, in the "salting out" of caseinogen and gelatin by sodium sulphate, the ratio of salt to water in the more protein-rich phase was less than in the watery phase—a similar relation to that found by de Bruyn [1900] in alcohol separation.

The same is true in the case of egg-albumin. An albumin solution was precipitated by ammonium sulphate and a complete analysis made of the original solution, the precipitate and the filtrate. In order to free the precipitate from adherent mother-liquor, it was not simply dried between filter paper, as done by Spiro, but placed between filter paper, surrounded by kieselguhr and submitted to a pressure of about 3 tons to the square inch. This pressure was, however, more than enough to squeeze out adherent mother-liquor, and actually removed some of the imbibed water of the protein-rich phase, for the concentration of salt in the liquid expressed, in the one case where it was collected and analysed, was only about half of that in the watery phase. The analyses given in Table VIII, where the results of these experiments are set out in detail, do not therefore express the composition of the two phases which were in equilibrium. The experiments prove, however, that the protein had appropriated some of the water for, notwithstanding this squeezing out of weak salt solution, the ratio salt to water in the compressed cake was considerably less than in the watery phase. See Table VIII.

This means that egg-albumin, like caseinogen and gelatin, dissolves or imbibes water just as alcohol does, and a mixture of protein, salt, water can be made to separate into two phases, either by the further addition of salt or of protein in the same way as a mixture of alcohol, salt, water separates on the addition of either salt or alcohol. In either case it amounts to increasing the concentration of salt in the watery phase.

To explain the influence of hydrogen ion concentration in the precipitation of egg-albumin, we must suppose that the electrical condition of the colloidal particles is a factor which modifies the ease with which they aggregate under the influence of the salt present. Hofmeister [1889] was influenced by a similar idea when he made parallel experiments with colloidal ferric hydroxide and egg-white, and Posternak [1901, 1 and 2], when investigating the precipitation of vegetable globulins, found that the efficiency of various electrolytes was influenced by the reaction of the suspension and presumably by the charge originally carried by the protein particles.

TABLE VIII.

Precipitation of pure egg-albumin by ammonium sulphate; composition of filtrate, pressed precipitate and press-liquor.

Exp.	Composition % ₀ by weight of			In original mixture	In pressed precipitate	In filtrate	In press liquor
I	Egg-albumin	9.56	64.65	0.84	—
	(NH ₄) ₂ SO ₄	23.35	29.83	26.67	—
	H ₂ O	67.08	6.39	72.49	—
	Salt present in 100 parts salt and water			25.82	17.6	26.9	—
II	Egg-albumin	12.48	63.69	0.59	—
	(NH ₄) ₂ SO ₄	22.60	29.91	27.04	—
	H ₂ O	64.92	8.47	72.37	—
	Salt present in 100 parts salt and water			25.82	22.1	27.2	—
III	Egg-albumin	9.47	73.57	1.58	0
	(NH ₄) ₂ SO ₄	24.09	22.04	27.66	15.2
	H ₂ O	66.42	6.39	70.76	84.8
	Salt present in 100 parts salt and water			26.62	22.47	28.1	15.2

The particles of proteins in acid or alkaline solutions carry respectively a positive or negative electric charge. Only at the iso-electric point, which is slightly to the acid side of the true neutral point in all cases hitherto investigated, does the charge disappear.

Salts like ammonium sulphate and sodium sulphate will cause separation into two phases, one protein-rich and the other protein-poor, whatever the charge upon the protein aggregates of the colloidal solution, if enough of the salt be added. Such phase separation is very materially assisted if the particles are positively charged, i.e. in solutions more acid than the iso-electric point. In the case of sodium chloride, indeed, acidification is necessary with many proteins.

From the analogy of the "salting out" of alcohols, phenols, etc. one must suppose that, by the gradual withdrawal of water from the protein aggregate by the salt, a critical dispersion point is reached when the surface tension at the interfaces causes the particles to run together. Supposing the particles are not iso-electric with the continuous phase, either from the original solution being acid or alkaline, or from the preferential adsorption of one of the ions of the electrolyte, the possession of charge will lower surface tension, so that, with charged particles, a higher concentration of salt will be required to arrive at the critical dispersion point. With negatively charged particles, in an alkaline solution, the charge cannot be neutralised by the more potent

ion SO_4^{--} which, if adsorbed, would still further increase the negative charge. We find that once the solution is more alkaline than the neutral point the amount of alkali added makes no difference¹ to the amount of salt required for precipitation, see Exp. III, Table VI and curve Fig. 5. The moment the reaction is made more acid than the iso-electric point and the protein particles carry a positive charge, this will at once be neutralised by the adsorption of SO_4^{--} ions. As these are in such high concentration they are apparently able to counteract in this way the maximum positive charge imposed upon the particles by the addition of acid (see Exp. III, Table VI, $\text{H}^+ = 0.007$ normal).

We venture to put forward the above interpretation from the analogous action of SO_4^{--} upon protein particles under conditions which permit of the demonstration of the existence of charge. In acid solution, protein particles, carrying a positive charge, have been shown to be sensitive to the anion of any electrolyte they may encounter. (Hardy [1900] for heated egg-white; Chick and Martin [1912] for denaturated serum proteins and egg-white; Chick [1913] for euglobulin and caseinogen.) Arguing from analogy we may suppose that in the case of egg-albumin the SO_4^{--} ion of ammonium sulphate will also be more readily adsorbed and any charge on the protein neutralised if the solution be originally on the acid side of the iso-electric point, i.e. if the protein particles carry a positive charge.

The statements detailed above have not actually been substantiated in case of pure egg-albumin, nor has the iso-electric point been determined. This has, however, been done in case of serum-albumin [Michaelis and Mostynski, 1910; Michaelis and Rona, 1910], and these two proteins otherwise display close similarity as regards the conditions of their solution or precipitation. We have not been able to put our interpretation to the direct test because it is impracticable to determine the charge carried by the particles of egg-albumin in the presence of excess of ammonium sulphate, nor under these conditions were we able to study the influence of addition of ions of varying valency. To test our hypothesis we therefore had

¹ In the case of Na_2SO_4 , which as regards the influence of acid behaves in an analogous manner to $(\text{NH}_4)_2\text{SO}_4$, excess of alkali (NaOH) favours precipitation, but, compared with the acid, a high concentration is required. In accordance with the explanation of the influence of reaction set forth above we presume that, while in acid solution the positively charged protein particles attract the SO_4 ion, in alkaline solution the Na ion of the sodium salt is preferentially absorbed. The electric charge on the particles is neutralised in both cases, but more readily in the first, owing to the greater potency of the SO_4 ion. With $(\text{NH}_4)_2\text{SO}_4$ no effect in solutions made alkaline with ammonia can be demonstrated, owing, presumably, to the low ionisation of $(\text{NH}_4)\text{OH}$, especially in the presence of excess of $(\text{NH}_4)_2\text{SO}_4$.

recourse to the expedient of withdrawing the water from the protein-water-combination by the addition of alcohol to the point where a surface tension is just manifest at the interfaces. In other words, the alcohol was added until the solution became opalescent but short of commencing precipitation, which could then be brought about by addition of a small amount of various electrolytes. Two sets of experiments were made; in one set the original protein solution was acid, and in the second set alkaline, and it was found, as was expected, that an electrolyte was efficient in causing precipitation of the protein in order of increasing valency of its anion in the first case and of its kation in the second. All solutions contained the same concentration of alcohol.

The results of a series of experiments with serum proteins are given in Table IX. In solution A (acid) salts containing divalent (ammonium sulphate) and trivalent (sodium citrate) anions caused precipitation in respective concentrations of 0.00055 and 0.00036 molar, whereas, in case of a monovalent anion (magnesium nitrate) about ten times the concentration was required. The valency of the kation was not without effect, but worked in the opposite direction; a small concentration of lanthanum nitrate (0.0007 molar) cleared up the original opalescence of the solution. For the same reason magnesium sulphate proved to be a much less efficient precipitant than ammonium sulphate.

An exactly converse set of results was obtained when the protein solution was originally made alkaline (B. Table IX). In this case lanthanum nitrate was the most powerful precipitant of all the salts tried and the sulphate of magnesium was much more effective than that of ammonium. At the same time a small concentration of sodium citrate (0.0007 molar) caused the solution to become clear.

With alkaline solutions higher concentration of lanthanum nitrate (0.0036 molar) prevented the formation of a precipitate, no doubt owing to the acquisition of a positive charge in excess of that needed to neutralise the negative one originally possessed. In acid solution an analogous result followed addition of sodium citrate to a concentration of 0.0007 molar.

Some experiments made with pure egg-albumin are set out in Table X. The original solution was acidified and after addition of sufficient alcohol to cause turbidity, determination was made of the concentration of a series of sodium salts necessary to cause a precipitate to form. The influence of increasing valency of the anion is very marked, sodium citrate and sulphate being respectively about 800 and 25 times as powerful in this respect as sodium chloride.

TABLE IX.

Precipitation of serum proteins by various electrolytes in presence of alcohol (almost to precipitation) at 0°.

Protein content=about 0.1 %.

A. In acid solution.

Salt	Conc. (molar)	Conc. (normal)	Degree of precipitation	Conc. (molar) required for com- plete precipitation
Na ₃ Cit	0.00007	0.00022	+	0.00036
	0.00036	0.0011	++	
	0.00071	0.0022	+	
	0.0036	0.011	-	
(NH ₄) ₂ SO ₄	0.00011	0.00022	+-	0.00055
	0.00055	0.0011	++	
	0.0055	0.011	++	
	0.055	0.11	++	
MgSO ₄	0.00055	0.0011	+	0.0011
	0.0011	0.0022	++	
	0.0055	0.011	++	
	0.055	0.11	++	
Mg(NO ₃) ₂	0.0011	0.0022	+-	0.0055
	0.0055	0.011	++	
La(NO ₃) ₃	0.00071	0.0022	-	
	0.0036	0.011	-	

B. In alkaline solution.

La(NO ₃) ₃	0.000071	0.00022	+-	0.00036
	0.00036	0.0011	++	
	0.00071	0.0022	++	
	0.0036	0.011	+	
Mg(NO ₃) ₂	0.00011	0.00022	+-	0.00055
	0.00055	0.0011	++	
	0.0055	0.011	++	
	0.055	0.11	++	
MgSO ₄	0.0011	0.0022	++	0.0011
	0.0055	0.011	++	
(NH ₄) ₂ SO ₄	0.0011	0.0022	+-*	No precipitation
	0.0055	0.011	+-*	
Na ₃ Cit	0.00071	0.0022	-	" "
	0.0036	0.011	-	

* Clearer than control solution containing alcohol only.

- =clear solution, clearer than the control, containing alcohol only.

- + = opalescent solution.

+

TABLE X.

Precipitation of pure egg-albumin, in acid solution, by sodium salts, in presence of alcohol almost to precipitation; influence of anions.

Protein content = 0.7 %.

Temperature, 0°.

Salt	Concentration required to cause precipitation in presence of alcohol (Molar)	Concentration necessary to commence precipitation in absence of alcohol (Molar)
Citrate (neutral)	0.00013	1.37
Phosphate	0.00016	2.50
Tartrate	0.003	1.60*
Sulphate	0.004	1.69
Acetate	0.09	—
Chloride	0.10	4.3*
Chlorate	0.35	—
Chromate	0.4*	1.71*

* Did not precipitate.

In the same Table are given the results of a series of experiments in which precipitation took place in absence of alcohol and it will be seen that no such influence of valency can be detected here. The relation of the various electrolytes, with the exception of sodium citrate, is that expressed by the Hofmeister series [1888] and doubtless conditioned by their relative water-drawing capacity. When the protein is on the point of being precipitated after the necessary water-withdrawal has taken place, the influence of the anion or kation of the electrolyte present can complete precipitation by neutralising a charge. This occurs for example when protein almost precipitated by ammonium sulphate is made slightly acid, or when to protein almost thrown out by alcohol a trace of an appropriate electrolyte is added. In the former case, as long as the solution remains alkaline, the effect of presence of ammonium sulphate with its divalent anion will rather be to increase the negative charge on the protein.

An additional piece of evidence in support of the view that the charge carried by the protein particles is an important factor in determining the concentration of salt requisite to occasion separation into two distinct phases, was obtained from studying the "salting out" of egg-albumin with calcium chloride. In this case the positive ion of the electrolyte is prepotent and we should expect it to act more efficiently if the solution containing the protein is made more alkaline than the iso-electric

point. This proved to be the case¹. It was found that in a solution containing 0.8 % protein and 37.7 % CaCl_2 an opalescence was developed on standing for about 2 hours at 18° when the hydrogen ion concentration was about 23×10^{-7} normal. In presence of a small concentration of $\text{Ca}(\text{OH})_2$, under otherwise similar conditions, the hydrogen ion concentration fell to about 0.05×10^{-7} normal. In this case almost all the protein was precipitated and only a trace remained in the filtrate.

Precipitation by calcium chloride differs from that by ammonium sulphate in the fact that the process is irreversible, and the precipitate is formed slowly and becomes insoluble. We were not able to make satisfactory experiments with other electrolytes of the same character, e.g. the nitrates and chlorides of magnesium and barium, because with egg-albumin precipitation is only very partial even in alkaline solution, but in these cases also, we were able to satisfy ourselves that alkalinity of the solution assisted the separation of a precipitate (protein-rich phase).

SUMMARY.

1. The precipitation of egg-albumin by ammonium sulphate is, as Spiro demonstrated to be the case with sodium sulphate, and caseinogen and gelatin, due to the separation of the system into a protein-rich phase and a watery phase, and to a certain extent is analogous with the salting-out of alcohol.

2. The first effect of concentrated salt is to withdraw water from the protein aggregates. A surface tension is in consequence developed at the interfaces, which causes the protein particles to aggregate, thus dividing the system into two distinct phases (precipitate and filtrate).

3. All three constituents of the system, viz. protein, water and salt, are present in each phase; the proportion, however, is different. The precipitate (protein-rich phase) contains relatively little water and salt, and the filtrate (watery phase) relatively little protein. Under appropriate conditions practically all the protein may be precipitated, only a trace remaining in the filtrate.

The two phases are in equilibrium, and any alteration in the amount of any one of their three constituents is followed by a change both in their composition and volume. Thus increase in concentration of salt or of protein

¹ Precipitation by calcium chloride is also assisted in cases where the solution is markedly acid, a comparatively large concentration of acid (HCl) being needed to demonstrate the phenomenon. The explanation is exactly analogous to that offered for the case of sodium sulphate, see footnote, p. 393, but in the reverse sense, much more acid being required to produce this effect than is the case with alkali.

is followed by a corresponding increase in the protein-rich phase (precipitate). Owing to rigidity of the latter readjustment is, however, slowly accomplished.

4. The relative volume of the two phases (amount of precipitate) is altered by varying the temperature.

5. The exact proportion of salt, protein and water at which phase separation (precipitation) occurs and the relative volume of the two phases (amount of the precipitate) is very sensitive to hydrogen ion concentration in the neighbourhood of the iso-electric point. In the case of $(\text{NH}_4)_2\text{SO}_4$, when the hydrogen ion concentration varied from 10^{-6} to 10^{-5} normal, the amount of the precipitate increased from a negligible amount to a maximum.

6. With CaCl_2 , in which the kation is prepotent, a similar effect was observed, but in the opposite direction.

7. An interpretation of the dominating influence of hydrogen ion concentration over this range is put forward and some experiments in support of it adduced.

The principles discussed in this paper must be borne in mind whenever salting-out is made use of in the fractionation of protein solutions and the purification of isolated fractions by re-solution and reprecipitation. Conclusions as to the homogeneity of proteins isolated by salt precipitation must also be reconsidered in the light of these results.

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XXVII. THE PREPARATION FROM ANIMAL TISSUES OF A SUBSTANCE WHICH CURES POLYNEURITIS IN BIRDS INDUCED BY DIETS OF POLISHED RICE.

PART I.

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(Received April 8th, 1913.)

Although several workers have isolated from various food-stuffs substances capable in small amount of curing polyneuritis in pigeons and have ascribed chemical formulae to them, attempts to prepare the active constituent in an amount sufficient for investigating its chemical constitution and properties have up to the present time been unsuccessful.

Funk [1911, 1913] has isolated from rice polishings, yeast, milk, and ox-brain substances curative in oral doses of 0.02 g. and possessing similar melting-points.

Edie, Evans, Moore, Simpson, and Webster [1912] have also prepared a substance from yeast curative in small amount, to which they have ascribed the formula $C_7H_{17}O_5N_2$, and Suzuki, Shimamura, and Odaki [1912] isolated an active substance containing nitrogen from rice-polishings in the form of a picrate, but they have not published any results of its analysis.

1. ISOLATION OF AN ANTI-NEURITIC SUBSTANCE FROM HORSE-FLESH.

In the present communication a method is described for preparing from horse-flesh a substance capable in minute amount of curing polyneuritis in pigeons.

The method employed was a modification of that described by Maclean [1912, 1] for the isolation and purification of the phosphatides of the kidney. The horse-flesh was minced, dried at 30° by means of an electric fan and ground, and the dry powder (weight 4000 g.) extracted at 37° with absolute alcohol on a shaking machine. The filtered extract was evaporated at 40° in

vacuo to remove the alcohol. The extract (weight 500 g.) was found to possess marked curative properties towards pigeons affected with polyneuritis, 4 g. being sufficient to bring about complete recovery within 24 hours. The extract was then treated with an excess of ether and the mixture allowed to stand in the cold room for 12 hours. The ether dissolved the fats and lipoids, but left undissolved a considerable amount of a white substance. This was washed thoroughly with ether and then tested on neuritic pigeons. Doses of 0.3 g. were sufficient to bring about complete recovery within 12 hours. This procedure therefore afforded a simple technique for separating a highly curative fraction from fats and lipoids and it was much more convenient than extracting the active substance by means of water.

The ether-soluble fraction was freed from ether by evaporating *in vacuo*. As much as 12 g. of the residue possessed only slight curative properties, while 2 g. of the lipoids separated from this fraction by means of acetone were inactive, but 7 g. were curative within 24 hours. It was thus possible to separate the bulk of the anti-neuritic substance from the alcoholic extract of horse-flesh by means of ether. These results support the explanation advanced by Maclean [1912, 2] for the curative properties of lecithin observed by several workers, namely, that the anti-neuritic substance is not a lipoid, but is present in ordinary lecithin as an impurity readily extractable therefrom by simple methods.

The ether-insoluble fraction was next treated with absolute alcohol, by which a large portion was dissolved. The insoluble residue contained both inorganic and organic material, but possessed no curative properties. The alcohol-soluble fraction however was strongly curative, and it was treated with excess of ether. This caused the separation of a yellow syrup (yield 50 g.) which was completely soluble in water and 0.2 g. of which was sufficient to cure pigeons affected with polyneuritis. The aqueous solution of the syrup was allowed to stand in a vacuum desiccator and a white crystalline substance gradually separated. This proved to be carnine and possessed no curative properties. The filtrate, which was highly curative, was next treated with finely powdered lead acetate, until there was no more separation of a flocculent precipitate, and the mixture was allowed to stand for 12 hours. The precipitate was filtered off, washed with water at 35°, decomposed with sulphuric acid, and the excess of acid removed by the careful addition of baryta. The filtered solution possessed no curative properties. The filtrate from the lead acetate precipitation was freed from lead by careful treatment with dilute sulphuric acid and was left very slightly acid. It possessed curative properties and was next treated with silver nitrate, which produced

a copious yellowish-white precipitate. This was filtered off, decomposed by hydrochloric acid and the resulting solution was nearly neutralised with soda and was found to be curative. The filtrate from the silver nitrate precipitation was also curative, but its content of active substance was completely precipitated by silver nitrate when baryta was added. By carrying out several animal experiments it was found that at least $3/5$ of the total amount of anti-neuritic substance present in the filtrate from the lead acetate precipitation was precipitated by the addition of silver nitrate only, and about $1/4$ was carried out of solution by the subsequent addition of baryta. The remaining $3/20$ was probably destroyed by the alkali. The residue obtained by the evaporation of the curative solution resulting from the decomposition of the first silver nitrate precipitate with hydrochloric acid was next extracted with chloroform. Only a small amount of substance was extracted and this possessed no curative properties. The anti-neuritic substance was therefore insoluble in chloroform. It was also found to be insoluble in benzene and ethyl acetate. The residue insoluble in chloroform was next extracted with absolute alcohol; a large amount was insoluble, but the curative substance was found to have been entirely dissolved, and 0.10 g. of the soluble fraction was sufficient to cure a pigeon affected with polyneuritis. This fraction was then extracted with acetone and a considerable amount was dissolved. The insoluble residue, which contained all the active substance, was dissolved in 50 % alcohol, and the solution allowed to stand in a desiccator for some days. A white substance gradually crystallised out, 0.06 g. of which administered to each of two pigeons ameliorated the symptoms of polyneuritis in a few hours and effected a complete recovery within 24 hours. The substance dissolved with moderate readiness in water, but was insoluble in absolute alcohol.

It was not possible to obtain a sufficient amount of the active substance to determine its chemical composition, and the work is therefore being repeated on a much larger scale. As, however, it was found [Cooper, 1913] that cardiac muscle contained considerably more anti-neuritic substance than voluntary muscle, ox-heart has been substituted for the horse-flesh and the results so far obtained justify this change.

Some experiments have also been carried out to ascertain if the anti-neuritic substance could be precipitated by means of ether from the fats and lipoids derived from other animal-tissues. It was found that considerable amounts of curative fractions could be obtained by this procedure not only from horse-flesh and ox-heart, but also from horse-kidney, beef, ox-brain and liver.

Only a small amount of material, however, was separated by ether from the alcoholic extract of egg-yolk, and its curative power was very feeble. The results indicate that this fractionation affords quite a general method for separating the anti-neuritic substance from the fats and lipoids of animal tissues.

2. THE ABSORPTIVE CAPACITY OF ANIMAL CHARCOAL FOR THE ANTI-NEURITIC SUBSTANCE.

Chamberlain and Vedder [1911, 1912] found that an extract of rice-polishings containing the anti-neuritic substance after being filtered through bone-black could no longer prevent polyneuritis in fowls and that the active substance could only be extracted to a small extent from the charcoal by water or alcohol. As these results are of great importance, the experiments have been repeated. Powdered animal charcoal was extracted three times with boiling water and then dried. A curative solution of known activity (prepared by dissolving the ether-insoluble fraction derived from an alcoholic extract of horse-flesh in water) was filtered slowly through a bed of the charcoal six times. The filtrate was still curative, but its activity was reduced to the extent of 30 %. Separate portions of the charcoal were then extracted with water and alcohol, and the extracts were found to be highly curative. The anti-neuritic substance was thus partially absorbed by charcoal and could be recovered from the latter by extraction with water or alcohol. It is seen that the results do not agree entirely with those obtained by Chamberlain and Vedder, but it is unlikely that the use of bone-black as an absorptive agent will be of much value for the isolation of the anti-neuritic substance.

3. THE EFFECT OF SULPHURETTED HYDROGEN UPON THE ACTIVE SUBSTANCE.

In a preliminary fractionation of the horse-flesh extract sulphuretted hydrogen was employed to decompose the lead and silver precipitates and to remove the excess of the metals from solution, and during these processes a rapid disappearance of the active substance occurred. By substituting acids for the sulphuretted hydrogen (sulphuric acid for lead, hydrochloric acid for silver) it was possible to conduct the operations with a much smaller loss of active material.

These facts suggested that sulphuretted hydrogen destroyed the anti-neuritic substance. Accordingly, the effect upon activity of passing this gas

through a curative solution for four hours was investigated, but no evidence was obtained of any destruction of the anti-neuritic substance. The diminished activity during the above manipulations would therefore appear to have been due to the absorption of the curative substance by the colloidal metallic sulphides.

4. THE EFFECT OF ALKALI UPON THE ACTIVE SUBSTANCE.

Several investigators have observed that a considerable loss of anti-neuritic substance occurs when chemical operations involving the use of alkali are employed in the fractionations. It was thought desirable to throw some light on this matter by quantitatively studying the effect of treatment with alkali upon the curative power of the substance.

To a solution of known activity ammonia was added until its concentration throughout the total volume of liquid reached 10 %. The mixture was kept in a closed flask at ordinary temperatures for 24 hours, the ammonia then removed by a current of air, and the curative power of the solution redetermined. It was found that 50 % of the anti-neuritic substance had become destroyed by contact with the alkali.

These results indicate that the isolation of the anti-neuritic substance in large amount is only likely to be effected by employing chemical methods that do not involve the use of alkali and sulphuretted hydrogen.

5. THE EFFECT OF ALKALOIDS UPON PIGEONS AFFECTED WITH POLYNEURITIS.

Strychnine was found [Cooper, 1913] to prolong the lives of birds affected with polynneuritis but to exert no curative action. It was accordingly of interest to investigate the action of other alkaloids. Experiments have been carried out with quinine, cinchonine, and cinchonidine. The substances were dissolved in a trace of hydrochloric acid and administered to the birds orally.

Doses of quinine ranging from 0.01 to 0.1 g. exerted a temporary curative action. In six cases the symptoms were ameliorated for about three days and then became once more acute, further administration of quinine having no effect. In two other cases however the neuritic symptoms completely disappeared within a few hours, and one of the birds remained free from polynneuritis for four days, whilst the other, receiving daily in addition to polished rice 0.025 g. of quinine, remained healthy for 10 days and then died exhibiting

symptoms merely of weakness and not of acute polyneuritis. When given in amounts exceeding 0.1 g. however quinine had no ameliorative effect, but actually appeared to hasten the fatal issue.

Daily doses of quinine ranging from 0.01 to 0.025 g. administered to several pigeons fed on polished rice did not prevent or delay the development of neuritic systems.

Cinchonine in doses varying from 0.05 to 0.1 g. like quinine effected a marked improvement in the condition of pigeons suffering from polyneuritis, but within 48 hours the birds again exhibited the acute neuritic symptoms and died notwithstanding further doses of the alkaloid. Cinchonidine on the other hand had no ameliorative effect at all.

As quinine and cinchonine are extracted from cinchona-bark by means of acid, it was thought that their curative action might possibly be due to contamination with traces of the anti-neuritic substance present in the plants. The active substance is readily destroyed by heat, so that some light could be thrown on this matter by observing whether the alkaloids retained their curative properties after strong heating. It was actually found that after being heated at 125° for six hours quinine no longer had any ameliorative effect upon neuritic pigeons. As the alkaloid is not chemically altered by such treatment, it would appear that its curative action is due to the presence as an impurity of a minute amount of the anti-neuritic substance.

6. THE EFFECT OF ALCOHOL UPON BIRDS FED ON POLISHED RICE.

The administration of small doses of alcohol (0.5 c.c. three times daily) to pigeons fed on polished rice had no measurable effect upon the period of time elapsing before the occurrence of symptoms of polyneuritis, and thus appeared not to influence the utilisation of the supply of anti-neuritic substance distributed in the tissues of the birds.

This is presumptive evidence that alcoholic neuritis is not caused by any diminished capacity on the part of the organism to utilise the anti-neuritic substance which might be expected to result from the disturbing effects of alcohol upon the metabolism of the nervous system.

SUMMARY.

1. A fraction rich in the anti-neuritic substance can be precipitated from the fats and lipoids (alcoholic extracts) of various animal tissues by means of ether.

2. A method based on this observation is described for isolating from horse-flesh a substance small amounts of which can cure polyneuritis in pigeons.

3. The substance is insoluble in absolute alcohol, benzene, chloroform, ether, and ethyl acetate, but is moderately soluble in water.

4. The substance is absorbed to some extent by animal charcoal and is readily destroyed by alkali. It is not inactivated by sulphuretted hydrogen, but disappears in large amounts during chemical operations in which colloidal metallic sulphides are formed.

5. Quinine and cinchonine exert a temporary curative action upon birds affected with polyneuritis. After being heated at 125° for 6 hours, however, quinine has no ameliorative effect, so that its curative properties would appear to be due to its contamination with traces of the anti-neuritic substance derived from the cinchona bark.

6. The administration of small doses of alcohol to birds fed on polished rice does not affect the period of time elapsing before the occurrence of polyneuritis, and thus appears not to influence the utilisation of the supply of anti-neuritic substance distributed in the tissues of the birds. This suggests that alcoholic neuritis does not result from a diminished capacity of the organism to utilise the anti-neuritic substance.

In conclusion I am glad to have this opportunity of expressing my indebtedness to Dr Hugh MacLean for drawing attention to the curative properties of the residues obtained by treating alcoholic extracts of voluntary muscle with ether and for much valuable help in the course of the investigation.

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ON THE PROTECTIVE AND CURATIVE PROPERTIES OF CERTAIN FOODSTUFFS AGAINST POLYNEURITIS INDUCED IN BIRDS BY A DIET OF POLISHED RICE.

PART II.

By E. A. COOPER, *Beit Memorial Research Fellow,
Lister Institute of Preventive Medicine.*

IN a previous communication (Cooper, 1913) the amounts of various raw foodstuffs, *e.g.* beef, heart-muscle, brain, fish, egg-yolk, lentils, and barley were set forth which were sufficient to prevent polyneuritis for a definite period in pigeons fed on polished rice.

It was found that, while (ox) cardiac muscle, egg-yolk, lentils, and barley were very efficient in preventing polyneuritis, the voluntary muscle of both ox and fish possessed only feeble anti-neuritic powers. Sheep-brain was less efficient than ox-heart, but more so than beef in preventing polyneuritis.

The relatively small efficiency of brain material suggests that either the anti-neuritic substances, although essential for the integrity of the nervous system, are not present therein in the active condition or they are combined in such a form that they are not readily absorbed from the alimentary canal.

It was also found that, although small amounts of brain material did not prevent polyneuritis, they were effective in checking the loss in weight that usually accompanies the disease. This fact supported the conclusion reached by Schaumann (1911) that the loss in weight was not merely due to the deficiency in polished rice of the anti-neuritic substances, but resulted largely from a secondary deficiency of other substances essential for the maintenance of body-weight.

To obtain more information as to the relative efficacy of various foodstuffs for preventing beri-beri and to ascertain further facts as to

the distribution of the anti-neuritic substances in animal tissues, I have made some more experiments with brain, liver, milk, cheese, nuts, and malt-extract and the results obtained are set forth in the present communication.

These experiments consisted in determining the daily amounts of the above foodstuffs which prevented polyneuritis in pigeons for a definite period. As pigeons develop symptoms of polyneuritis in about three weeks when fed exclusively on polished rice, the standard time selected was 50 days.

Pigeons received daily by artificial feeding constant rations of polished rice equal to 1/20th their initial body-weight and rations different for each bird of the tissue under investigation. All the tissues were thoroughly minced before being fed to the pigeons.

In this way it was possible to determine a maximum daily amount insufficient to prevent polyneuritis for 50 days and a minimum amount sufficient for this purpose. The suitability of the various diets for the maintenance of body-weight was also studied, the birds being weighed weekly for this purpose.

I. *Control experiments with polished rice.*

Seven pigeons were fed artificially on polished rice, the daily ration being 1/20th their initial body-weight. The results of the experiments are set forth below.

TABLE I.

	Pigeon 326	Pigeon 327	Pigeon 328	Pigeon 329
Effect of diet on pigeons	Symptoms of polyneuritis appeared on 23rd day	Symptoms of polyneuritis appeared on 25th day	Symptoms of polyneuritis appeared on 20th day	Symptoms of polyneuritis appeared on 23rd day
Change in weight by end of exp.	- 26 %	- 24 %	- 17 %	- 22 %
	Pigeon 330	Pigeon 133	Pigeon 134	
Effect of diet on pigeons	Symptoms of polyneuritis appeared on 22nd day	Symptoms of polyneuritis appeared on 22nd day	Symptoms of polyneuritis appeared on 17th day	
Change in weight by end of exp.	- 16 %	- 22 %	+ 3 %	

The results indicate that the birds developed symptoms of polyneuritis in about three weeks and at the end of that time had usually lost considerably in weight.

II. *Ox-brain.*

TABLE II.

Series I. (a) Cerebellum (water-content 80 %).					
	Pigeon 10	Pigeon 11	Pigeon 12	Pigeon 13	
Daily ration of natural tissue	4 gms.	5 gms.	7 gms.	9 gms.	
Effect of diet on pigeon	Symptoms of polyneuritis on 21st day	Still healthy on 31st day	Still healthy on 31st day	Still healthy on 31st day	
Change in weight by end of exp.	0	- 9 %	0	- 12 %	
(b) Cerebrum (water-content 80 %).					
	Pigeon 6	Pigeon 7	Pigeon 8	Pigeon 9	
Daily ration of natural tissue	4 gms.	5 gms.	7 gms.	9 gms.	
Effect of diet on pigeon	Symptoms of polyneuritis on 22nd day	Still healthy on 31st day	Still healthy on 31st day	Still healthy on 31st day	
Change in weight by end of exp.	- 20 %	- 19 %	- 9 %	- 4 %	
Series II. (a) Cerebellum.					
	Pigeon 340	Pigeon 341	Pigeon 342	Pigeon 343	Pigeon 344
Daily ration of natural tissue	3 gms.	4 gms.	6 gms.	9 gms.	12 gms.
Effect of diet on pigeon	Symptoms of polyneuritis on 24th day	Symptoms of polyneuritis on 34th day	Weakness in limbs on 46th day	Died on 39th day with symptoms of weakness	Still healthy after 50 days
Change in weight by end of exp.	- 15 %	- 17 %	- 6 %	- 8 %	- 5 %
(b) Cerebrum.					
	Pigeon 345	Pigeon 346	Pigeon 347	Pigeon 348	Pigeon 349
Daily ration of natural tissue	3 gms.	4 gms.	6 gms.	9 gms.	12 gms.
Effect of diet on pigeons	Symptoms of polyneuritis on 30th day	Weakness in limbs on 46th day	Still healthy on 50th day	Still healthy on 50th day	Still healthy on 50th day
Change in weight by end of exp.	- 25 %	- 20 %	- 18 %	+ 5 %	- 17 %

The results indicate that the amounts of cerebrum and cerebellum required to prevent the *acute* symptoms of polyneuritis in pigeons fed on polished rice were about equal, but, while 6 gms. of cerebrum were sufficient to keep the birds healthy and strong for 50 days, 12 gms. of cerebellum were necessary to prevent signs of weakness in the later days of the experiment. Cerebrum therefore appeared to contain

a somewhat greater available amount of the anti-neuritic substances than cerebellum. Notwithstanding this however there was a tendency for cerebellum to be somewhat more effective than cerebrum in reducing the loss in weight resulting from the polished rice diet. These observations support the conclusion reached by Schaumann (1911) that the loss in weight accompanying polyneuritis is not entirely an effect of the malnutrition necessarily resulting from the deficiency in the diet of the anti-neuritic substances, but must be largely due to a secondary deficiency in polished rice of substances essential for the maintenance of body-weight. This view was also supported by the fact recorded in the previous communication (1913) that the addition of a small amount of sheep-brain to the polished rice diet, although ineffective in preventing polyneuritis, was sufficient to reduce the loss in weight to a considerable degree. On comparing the results obtained with ox- and sheep-brain however it is found that the former is less efficacious than the latter in this respect, although somewhat more potent in preventing polyneuritis. No explanation of this can at present be advanced.

III. *Or-liver.*

(*Water-content 70 %.*)

TABLE III.

	Pigeon A	Pigeon 1	Pigeon 2	Pigeon B	Pigeon 3
Daily ration of natural tissue	1 gm.	2 gms.	3 gms.	4 gms.	5 gms.
Effect of diet on pigeons	Symptoms of polyneuritis on 8th day	Symptoms of polyneuritis on 47th day	Still healthy on 50th day	Still healthy on 35th day	Still healthy on 31st day
Change in weight by end of exp.	- 9 %	- 13 %	- 8 %	- 6 %	- 11 %
	Pigeon 4	Pigeon 5	Pigeon 83	Pigeon 84	Pigeon 85
Daily ration of natural tissue	7 gms.	12 gms.	3 gms.	4 gms.	5 gms.
Effect of diet on pigeons	Still healthy on 31st day	Still healthy on 31st day	Still healthy on 50th day	Still healthy on 50th day	Still healthy on 50th day
Change in weight by end of exp.	- 3 %	- 3 %	- 28 %	- 13 %	0
	Pigeon 86	Pigeon 87	Pigeon 88		
Daily ration of natural tissue	7 gms.	9 gms.	12 gms.		
Effect of diet on pigeons	Still healthy on 50th day	Still healthy on 50th day	Still healthy on 50th day		
Change in weight by end of exp.	- 12 %	- 12½ %	0		

The results indicate that 3 gms. of ox-liver daily were sufficient to prevent polyneuritis in pigeons for 50 days, and 2 gms. delayed the development of the disease until the 47th day of the experiment. The richness of liver in the anti-neuritic substances suggested that the tissue might even be effective in curing polyneuritis. Accordingly, minced ox-liver was dried at 30° C. by an electric fan, ground, and the dry powder emulsified with water and administered to neuritic pigeons. It was found that 7 gms. of dried liver (21 gms. liver in natural condition) rapidly cured the pigeons and prevented the reappearance of the disease for one week, while 4 gms. ameliorated the symptoms but could not effect complete recovery. Cod-liver oil in doses ranging from 2 to 8 gms. however possessed no curative properties.

IV. *Cow's milk.*

(Water-content 87 %.)

Vedder and Clark (1912) found that of four fowls fed on polished rice and 5 c.cs. of fresh cows' milk daily two developed symptoms of polyneuritis in a short time, while the remaining two were healthy even after two months.

In the following experiments the fresh milk was well shaken in a machine immediately before use and in the case of the larger rations of milk the birds were fed four times daily.

TABLE IV.

	Pigeon 69	Pigeon 70	Pigeon 71	Pigeon 72
Daily ration of milk	2 c.cs.	3 c.cs.	4 c.cs.	6 c.cs.
Effect of diet upon birds	Severe weakness in limbs on 38th day	Symptoms of polyneuritis on 18th day	Symptoms of polyneuritis on 11th day	Severe weakness in limbs on 38th day
Change in weight by end of exp.	- 24 %	- 24 %	- 6 %	- 26 %
	Pigeon 74	Pigeon 57	Pigeon 52	Pigeon 53
Daily ration of milk	12 c.cs.	15 c.cs.	20 c.cs.	20 c.cs.
Effect of diet upon birds	Symptoms of polyneuritis on 18th day	Weakness in limbs on 16th day	Symptoms of polyneuritis on 33rd day	Symptoms of polyneuritis on 36th day
Change in weight by end of exp.	- 12 %	- 6 %	- 12 %	- 26 %
	Pigeon 54	Pigeon 55	Pigeon 138	Pigeon 153
Daily ration of milk	35 c.cs.	35 c.cs.	10 c.cs.	30 c.cs.
Effect of diet upon birds	Symptoms of polyneuritis on 49th day	Symptoms of polyneuritis on 54th day	Symptoms of polyneuritis on 11th day	Symptoms of polyneuritis on 19th day
Change in weight by end of exp.	- 20 %	- 12 %	- 18 %	- 16 %

It is thus seen that cow's milk possessed only feeble anti-neuritic properties, as much as 35 ccs. daily merely delaying the development of polyneuritis until about the 50th day of the experiment. The birds receiving diets of polished rice and milk also suffered considerable loss in weight.

V. *Nuts (Husked filberts).*

(Water content 4%.)

TABLE V.

	A		B	
	Pigeon 264	Pigeon 265	Pigeon 267	Pigeon 268
Daily ration of nuts	1 gm.	1 gm.	2 gms.	2 gms.
Effect of diet on pigeon	Symptoms of polyneuritis on 20th day	Symptoms of polyneuritis on 36th day	Still healthy on 50th day	Still healthy on 50th day
Change in weight by end of exp.	- 25 %	- 10 %	- 14 %	- 8 %
	C		D	
	Pigeon 269	Pigeon 270	Pigeon 271	Pigeon 272
Daily ration of nuts	3 gms.	3 gms.	5 gms.	5 gms.
Effect of diet on pigeon	Still healthy on 50th day	Still healthy on 50th day	Still healthy on 50th day	Still healthy on 50th day
Change in weight by end of exp.	0	- 11 %	+ 7 %	+ 13 %
	E			
	Pigeon 273	Pigeon 274		
Daily ration of nuts	7 gms.	7 gms.		
Effect of diet on pigeon	Still healthy on 27th day	Still healthy on 27th day		
Change in weight by end of exp.	+ 10 %	+ 8 %		

The results indicate that the daily addition of 2 gms. of husked filberts to the polished rice diet was sufficient to prevent polyneuritis in pigeons. For the maintenance of body-weight 5 gms. of nuts daily were necessary. As it was previously found (Cooper, 1913) that 3 gms. of lentils or 4 of husked barley were required to prevent polyneuritis, it appears that nuts are somewhat superior to these foodstuffs in anti-neuritic power.

VI. *Cheese.*

Finely-ground cheddar cheese (water-content 30 %) was employed in the experiments.

TABLE VI.

	A		B	
	Pigeon 275	Pigeon 276	Pigeon 277	Pigeon 278
Daily ration of cheese	1 gm.	1 gm.	2 gms.	2 gms.
Effect of diet on pigeon	Symptoms of polyneuritis on 14th day	Symptoms of polyneuritis on 20th day	Symptoms of polyneuritis on 31st day	Died on 38th day with symptoms of weakness.
Change in weight by end of exp.	- 8 %	- 17 %	- 10 %	- 19 %
	C		D	
	Pigeon 279	Pigeon 280	Pigeon 281	Pigeon 282
Daily ration of cheese	4 gms.	4 gms.	8 gms.	8 gms.
Effect of diet on pigeon	Symptoms of polyneuritis on 22nd day	Died on 38th day with symptoms of weakness	Symptoms of polyneuritis on 15th day	Died on 28th day with symptoms of weakness.
Change in weight by end of exp.	- 18 %	- 10 %	0	0

The results indicate that the addition of as much as 8 gms. of cheese daily to the polished rice diet failed to prevent polyneuritis. Thus, while 35 c.cs. of fresh milk daily (Table IV) delayed the appearance of polyneuritis until the 50th day of the experiment, the equivalent amount of cheese (3.5 gms.)¹ and even more than twice this amount had no preventive effect. Possibly the anti-neuritic substances are destroyed during the process of cheese-ripening or they may be less readily absorbed from cheese than from milk.

VII. *Malt Extract.*

Experiments were carried out to ascertain whether malt extract possessed curative properties against polyneuritis. Three samples of the extract were employed. Two of them were found to exert a rapid curative action upon neuritic pigeons, but a third sample even in large doses had no effect. The results of the experiments are briefly set forth below.

Sample I (Water-content 24 %)

Minimum curing dose = 5 gms.

„ II (Water-content 27 %)

Minimum curing dose = 7 gms.

„ III (Water-content 18 %)

10 gms. had no curative action.

¹ 100 parts of milk yield approximately 10 parts of cheese.

Discussion of Results.

In the following table the minimum amounts of the various foodstuffs required to prevent polyneuritis in pigeons fed on polished rice are compared.

Foodstuffs	Amounts necessary to prevent polyneuritis	
	In terms of natural foodstuff	In terms of dry-weight
Ox voluntary muscle (1)	20 gms.	5.0 gms.
Ox cardiac muscle (1)	5 "	1.7 "
Ox cerebrum	6 "	1.2 "
Ox cerebellum	12 "	2.4 "
Ox liver	3 "	0.9 "
Cow's milk	> 35 "	> 3.5 "
Sheep cerebrum (1)	8 to 15 "	1.6 to 3 "
Fish voluntary muscle (1)	> 10 "	> 2 "
Egg-yolk (1)	3 "	1.5 "
Lentils (dry) (1)	—	3 "
Barley (husked) (1)	—	4 "
Nuts (husked filberts)	—	2 "
Cheese	> 8 "	> 5.6 "

(1) Cooper, 1913.

The results indicate that the various ox-tissues are not of equal anti-neuritic power, liver being most effective in preventing polyneuritis, then cardiac muscle and cerebrum, next cerebellum, and least effective, voluntary muscle and cow's milk. This order still obtains when the results are expressed in terms of dry material.

Liver is thus considerably more efficient in preventing polyneuritis than either cerebrum or cerebellum. In the natural condition cardiac muscle is about as efficient as cerebrum, but somewhat more so than cerebellum; on comparing the dried materials however heart-muscle is of smaller anti-neuritic efficacy than cerebrum, but still retains its superiority to cerebellum.

Before attempting to draw conclusions from the above results as to the actual distribution of the anti-neuritic substances amongst animal tissues and fluids, however, it is necessary to determine to what extent the various food-materials are absorbed from the alimentary canal of birds. In man egg-yolk and voluntary muscle (both of ox and fish) can be almost completely absorbed, but liver and cardiac muscle, owing to their denser structure, are less readily digested (Hutchinson, 1900). As however in my experiments these tissues were well minced, there is no reason to suppose that absorption would be less complete. Brain material, on the other hand, is imperfectly absorbed, as much as 40%.

appearing in the faeces, and this may explain its relatively small efficacy in preventing polyn neuritis in birds.

I have not determined the proportion of these various foods absorbed by pigeons. It would involve a very large number of experiments to obtain results of any value, but experiments to determine the proportion absorbed when brain in various amounts is fed to birds are in progress.

The whole content of anti-neuritic substances is not absorbed even from a normal diet of grain, as it was possible to detect the presence of these substances in the excreta of a hen fed on maize, barley, and buckwheat. The active substances could also be detected in the faeces of a rabbit fed on white bread and cabbage. The excreta of the bird were collected daily for a fortnight and dried at 30° C. by an electric fan. The total amount of dry material obtained was 150 gms. This was extracted repeatedly at 35° C. with absolute alcohol in a shaking machine, the filtered extract concentrated *in vacuo* and freed from alcohol. The residue was a black gummy substance weighing 6½ gms. Three gms. of this, equivalent to one week's excretion, administered orally to a pigeon affected with polyn neuritis rapidly exerted a complete curative action and the bird again fed on polished rice remained well for a week. One gram of the extract, equivalent to two days' excretion, improved the condition of other neuritic birds, but did not effect complete recovery. The rabbit's faeces were collected daily for a week and dried at 30° C. as above. The total amount of dry material was 75 gms. This was extracted with alcohol, the procedure being similar to that employed in the case of the bird's excreta, and one half of the total amount of dry alcoholic extract, equivalent to about three days' excretion, rapidly cured a pigeon affected with polyn neuritis, but within twenty-four hours the bird again became ill.

The anti-neuritic substances present in the excreta may be derived not merely from the diet, but to some extent possibly from the bacteria growing in the large intestine. Yeast is known to be particularly rich in the active substances, so that it may be reasonably supposed that these substances are also synthesised by bacteria.

Experiments carried out to ascertain whether *B. coli* contains any considerable amount of anti-neuritic substance have been made but the extract from 2 gms. of bacteria was without effect. It is evident that the amount contained is much less than in yeast.

The results, so far, afford indications as to the composition of diets suitable for the prevention of beri-beri.

Of animal tissues heart-muscle, liver, and egg-yolk are much more valuable for this purpose than voluntary muscle of either fish or ox and, if reckoned as dry weight, are somewhat superior even to lentils, nuts, and barley, which are suitable vegetable foodstuffs to supplement the polished rice diet. The small value of flesh as a prophylactic against polyneuritis which emerges from my experiments on birds is borne out by practical experience.

According to Van Leent (1880) prior to 1876 the native crews of the Dutch East Indian Navy received a diet of polished rice (75 %) and meat or fish (25 %) and suffered considerably from beri-beri, while the European crews whose dietary contained in addition beans, peas, potatoes, and greens were almost free from the disease. Subsequently the native crews drew the same rations as the Europeans and as a result of this change there was a great fall in the number of beri-beri cases.

In 1902 and 1903¹ the native troops in the Philippines owing to the prevalence of cholera were prevented from obtaining a supply of vegetables from the markets and were consequently restricted to a diet of polished rice and meat. Soon after this change beri-beri broke out and a large proportion of the Company were affected, although for a whole year before the troops had been in excellent health.

The substitution of heart-muscle and liver for ordinary flesh in the mixed diets employed in localities where beri-beri occurs would thus be a distinct improvement, as, not only are the former tissues when properly prepared as nutritious as voluntary muscle, but they also contain higher available concentrations of the anti-neuritic substances.

SUMMARY.

1. Pigeons fed on daily rations of polished rice equal to 1/20th their initial body-weight develop symptoms of polyneuritis in about three weeks and usually lose considerably in weight.

2. The efficacies of various ox-tissues for preventing polyneuritis have been determined, and the tissues arranged according to their anti-neuritic powers are in the following descending order: liver, cardiac-muscle, cerebrum, cerebellum, voluntary muscle, and (cows') milk.

3. Alcoholic extracts of the excreta of a hen fed on unpolished grain and of the faeces of a rabbit fed on bread and cabbage cured polyneuritis in pigeons. The whole content of anti-neuritic substances

¹ Report Surg.-Gen. Army, U.S.A. 1902-1903, p. 69.

present in the dietary was therefore not absorbed or else some amount is synthesised by the bacteria of which the faeces consisted to a considerable extent. No conclusions can consequently be drawn as to the actual distribution of the active substances in the animal body, until the extent to which the various tissues are absorbed from the alimentary canal of birds has been determined.

4. Nuts (husked filberts) are very efficient in preventing polyneuritis, being even superior to lentils and husked barley. Cheddar cheese, on the other hand, even in considerable amount, has no preventive effect.

5. Malt extract taken from two different samples readily cured polyneuritis in pigeons. A third sample however even in large doses had no curative action.

6. For the prevention of beri-beri egg-yolk, heart-muscle, liver, nuts, barley, and lentils can be recommended as suitable foodstuffs with which to supplement the polished rice diet. As meat (voluntary muscle) has been frequently found to be ineffective in preventing epidemics of beri-beri, its replacement by heart and liver in mixed diets would be a considerable improvement, because, not only are these tissues when suitably prepared as nutritious as voluntary muscle, but they also contain the anti-neuritic substances in much higher concentration.

I desire to express my best thanks to Professor C. J. Martin, F.R.S., for valuable help and criticism.

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XXXII. THE CURATIVE ACTION OF AUTOLYSED YEAST AGAINST AVIAN POLYNEURITIS.

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(Received May 5th, 1914.)

In a previous communication [Cooper, 1913] a method was described for the preparation from voluntary muscle of a substance, small amounts of which readily cured polyneuritis in pigeons. The method employed however was not only expensive, but the yield of active substance was very small, so that an attempt has since been made to discover a less costly process of preparing a non-toxic strongly curative substance suitable for use in the treatment of human beri-beri.

Yeast has for some time been known to be very effective in curing polyneuritis in birds, its curative action having been first observed by Schaumann [1910], and Cooper and Funk [1911] subsequently showed that the yeast still retained its curative properties after hydrolysis with 20 % sulphuric acid for 24 hours.

As yeast contains a powerful proteolytic ferment and is easily obtainable in the East, it was thought that possibly a suitable curative solution could be prepared from it by autolysis.

325 gms. of pressed brewers' yeast was accordingly placed in a flask in the hot room (35° C.) for 36 hours, and the yeast was rapidly converted into a brown fluid. This was filtered, and the residual cell-debris washed with a little water. The total volume of the combined filtrate was 270 cc.; 3 cc. of the liquid readily cured pigeons (300 gms.) affected with polyneuritis in 3 to 12 hours, and even 1—2 cc. improved the condition of the birds. As from 3 to 6 gms. of pressed yeast was necessary to bring about complete recovery, it is seen that the solution resulting from autolysis was at least as strongly curative as the original yeast.

A solution of similar potency could be obtained by the addition of 95 per cent. alcohol to the autolysed yeast, but, apart from the fact that the alcohol coagulated the cell-debris and so facilitated filtering, this method had no advantage over the one described above.

By treating the alcohol-free filtrate with basic lead acetate, a copious precipitate was obtained, and the active substance passed almost entirely into the filtrate. In this way much of the colouring matter could be removed, and the highly curative filtrate, although not superior to the original solution therapeutically, can be so readily obtained in large amount that it should be suitable raw material for fractionation with a view to the isolation of the anti-neuritic substance.

Five kilos. of pressed yeast were next autolysed as before for 36 hours, and the filtered solution measured 4500 cc. Three cc. of this readily cured pigeons affected with polyneuritis in a few hours, and the solution stored in the cold room retained its curative power for at least 8 weeks. Birds were then given orally large volumes of this active solution to ascertain if it exerted a toxic action, and the following results were obtained:—

Bird	Weight	Dose	Effect
Pigeon	300 gms.	30 cc. (15 cc. twice in 8 hrs.)	} Still healthy 1 week afterwards.
"	300 "	30 " " "	
Chicken	1800 "	100 cc. (50 cc. " " "	
"	500 "	36 cc. (18 cc. " " "	

The results show that as much as 10 times the curing dose (3 cc.) given to pigeons had no injurious effect, and even 100 cc. given to the chickens did no harm.

Some experiments were also carried out to ascertain whether a strongly curative solution could be as easily prepared when the yeast was first air-dried at 20° C. To 75 gms. of the dry preparation, which had been stored for six weeks in a dry tin, 250 cc. of water was added, and the yeast allowed to autolyse at 35° C. for 48 hours. The mixture was then filtered, and the insoluble matter washed with water, the combined filtrates measuring 250 cc. Three cc. of the solution again rapidly cured neuritic pigeons. As 1-2 gms. of the dried yeast were required to effect complete recovery, it is seen that the solution was quite as strongly curative as the material from which it was derived. 0.5 cc. of the solution injected subcutaneously rapidly cured a pigeon, and 1-2 cc. had no toxic action.

Air-dried yeast retained its marked anti-neuritic properties even after storage in a dry tin for two years, and after four months' storage still readily autolysed when mixed with water and kept at 35° C.

By autolysing yeast it is thus possible to prepare a highly active solution which being non-toxic and inexpensive should be suitable for the treatment of human beri-beri on a large scale. Owing to the stability of the anti-neuritic substance and proteolytic ferment contained in yeast, as shown above, the yeast can be air-dried, stored under dry conditions, and autolysed as required.

SUMMARY.

1. By the autolysis of brewers' yeast a solution can be obtained which possesses as marked curative properties towards avian polyneuritis as the original yeast.

2. The solution retains its curative power for at least eight weeks and when given orally to birds in doses 10 times as great as the minimum curing dose has no toxic action.

3. Air-dried yeast retains its curative power after storage for two years in a dry tin, and still autolyses after storage for four months.

CONCLUSION.

The autolysis of brewers' yeast should afford a simple inexpensive method of preparing a non-toxic solution suitable for the oral treatment of human beri-beri.

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STUDIES ON BERI-BERI. VII. CHEMISTRY OF THE
VITAMINE-FRACTION FROM YEAST AND RICE-
POLISHINGS. By CASIMIR FUNK, *Beit Memorial
Research Fellow.*

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IN my preliminary communication on this subject⁽¹⁾ I have described the separation of the crude curative, crystalline fraction from yeast into three substances. These have now been carefully purified and investigated. This new line of investigation was then extended to rice-polishings and similar results were obtained. In the earlier paper it was suggested, that the curative power of the crude fraction might be the resultant of the individual actions of these substances. As this investigation shows this is really the case, at least two substances being necessary to effect a cure. It seems however that the action of the isolated substances is slightly diminished as compared with the original extract or the original crude fraction. The early work of Gryns⁽²⁾ showed that the substances in question are unstable, extracts heated at 130° completely losing their curative power. During this investigation it was observed, that the vitamins actually decompose during the fractionation and this apparently accounts for the diminished curative power of the products isolated. At present we are unable to avoid this decomposition, our methods being too rough for this purpose. A good deal of work is still required to solve the difficult chemical problem of beri-beri.

It is evident that this can only be done by systematic work, and the present contribution throws some additional light on the question. The vitamine-fraction from yeast, as was stated above⁽¹⁾, can be obtained in the crystalline state and can be used for therapeutical purposes with very good effect. The chemical investigation of this fraction is nearly completed. It consists of three different substances, two of which seem to play a very important part in the process of curing. Each of these substances has been investigated chemically and pharmacologically¹.

¹ Prof. Cushny, to whom I wish to express my best thanks, kindly performed some experiments and showed that these substances possessed no marked action on blood-pressure, respiration and heart.

The chemical investigation was only rendered possible by using the excellent method of micro-combustion of Pregl.

The fractionation of rice-polishings has so far yielded only two substances which are now being investigated. All these substances belong to the same group of compounds and show very similar chemical properties. One of them has been obtained both from yeast and from rice-polishings. This appears to be nicotinic acid, which was isolated for the first time from rice-polishings by Suzuki, Shimamura and Odake⁽³⁾. The occurrence of nicotinic acid in both curative fractions, along with the results of actual experiments performed, suggests that this acid plays a certain part in the process of curing.

Derivatives of nicotinic acid are frequently found in plants, forming alkaloids like trigonelline, arecaine, etc. The new base recently isolated by me from lime-juice⁽⁴⁾ appears to be also a derivative of nicotinic acid, but this point requires further investigation.

The action of the substances described in this communication is summarised in a table at the end of this paper.

Investigation of yeast extract.

The extract was prepared from 100 kg. of dried yeast, following the method already described in one of my early papers⁽⁵⁾ and only the silver and baryta fraction (vitamine-fraction) carefully investigated. Other fractions obtained during this investigation will be described at a later date.

The whole of the vitamine-fraction amounted to 2.5 gm. The product was crystalline and melted at 210° (uncorr.). The pharmacological action of this product has been described in my previous paper⁽⁶⁾. This product gives a very strong blue reaction with the uric acid and phenol reagent of Folin and Macallum, junr.^{(6)¹}

The initial product was recrystallised from dilute alcohol and yielded 1.6 gm. which crystallised in needles, which melted at 225° (corr.). This product still showed both the reactions mentioned above and was recrystallised three times. The second crystallisation melted at 229° (corr.) and no longer gave the reaction. Further recrystallisation did not raise the melting point. A sample of the substance previously isolated from yeast was compared with this product, and was found to melt at a distinctly higher temperature (233° corr.). When mixed together in equal proportions they melted at 229°. Both substances

¹ Every crude curative fraction so far obtained gives these reactions. This point is being investigated in collaboration with A. B. Macallum, junr.

were identical in crystalline shape, solubility and reactions. The substance is a strong acid and does not show any of the known colour reactions. It is very insoluble both in cold and warm water. Because of this insolubility it could not be decided whether the substance is optically active or not. A 0.74% solution did not show any appreciable rotation, other solvents could not be used, as the substance is entirely insoluble in all of them.

The substance gave the following figures on analysis (method of Pregl). Dried in vacuum at 100°.

3.267 mg.	gave 6.63 mg. CO ₂ and 1.05 mg. H ₂ O ;	55.35 % C and 3.60 % H
4.124 "	8.425 " " 1.36 " ;	55.72 " " 3.69 "
4.256 "	8.66 " " 1.36 " ;	55.50 " " 3.58 "
3.048 "	0.373 cm. ³ N at 15.5° and 710 mm. ;	13.53 % N
3.627 "	0.430 " " 14° " 712 " ;	13.21 "
3.286 "	0.401 " " 18° " 705 " ;	13.28 "

These figures correspond to the formula: C₂₄H₁₉O₉N₅ (521.24) which requires: 55.25 % C, 3.68 % H and 13.44 % N.

When dried at 100° a small sublimate appears in the drying tube. Analyses were therefore also made with the substance dried only in a vacuum desiccator over sulphuric acid and the following figures obtained:

3.733 mg.	gave 7.775 mg. CO ₂ and 1.185 mg. H ₂ O ;	56.80 % C and 3.55 % N
3.538 "	7.35 " " 1.195 " ;	56.66 " " 3.78 % H
3.174 "	0.372 cm. ³ N at 17° and 702 mm. ;	12.74 % N

These figures correspond to the formula: C₂₅H₂₁O₉N₅ (547.26) which requires: 57.01 % C, 3.87 % H and 12.81 % N.

The substance loses apparently on drying a certain amount of product rich in carbon. This may account for the difference in melting point, mentioned above, as one of these substances was (the recently obtained one) dried at 100°.

0.1328 gm. of the substance requires (Kjeldahl) 4.0 c.c. N/10 H₂SO₄. Found 4.21 % N. According to this only one-third of the nitrogen can be estimated by the Kjeldahl method.

0.0373 gm. to become neutral to phenolphthalein requires 2.8 c.c. of N/10 KOH. Calculated from the formula C₂₄H₁₉O₉N₅ for a tetrabasic acid 2.8 c.c.

The filtrate from the first substance gives a very strong reaction with the uric and phenol reagents. By fractional crystallisation 0.4 gm. of a substance was obtained, which melted at 210°. The substance was recrystallised twice from dilute alcohol, the melting point rising to 222-3° (uncorr.). It forms microscopical needles, and is

slightly more soluble in water than the first substance. When purified, the substance does not give the uric acid reaction, but still gives the phenol reaction, although the Millon reaction is negative. For analysis one sample of the substance was dried in a vacuum desiccator over sulphuric acid, another at 115° in vacuo.

Dried over sulphuric acid :

3.559 mg. gave 7.745 mg. CO_2 and 1.21 mg. H_2O ; 59.35 % C and 3.80 % N
3.445 ,, 0.375 cm^3 N at 17° and 706 mm.; 11.90 % N

Dried in vacuo at 115° :

3.694 mg. gave 7.99 mg. CO_2 and 1.22 mg. H_2O ; 58.99 % C and 3.70 % H

Dried in vacuo at 100° :

4.172 mg. gave 9.065 mg. CO_2 and 1.535 mg. H_2O ; 59.26 % C and 4.12 % H
2.716 ,, 0.295 cm^3 N at 15° and 711 mm.; 12.04 % N

Calc. for $\text{C}_{23}\text{H}_{18}\text{O}_7\text{N}_4$ (462.22). Calc. for $\text{C}_{29}\text{H}_{23}\text{O}_9\text{N}_5$ (585.28).

59.71 % C	59.46 % C
3.84 % H	3.96 % H
12.12 % N	11.97 % N

The filtrate from the second substance still showed the uric acid reaction, and when further concentrated yielded 0.45 gm. of colourless silky needles in contrast to the previous substances which appeared slightly grey. After recrystallisation the melting point rose to 235° (uncorr.), at which point it remained stationary. It is fairly soluble in cold water, and after recrystallisation loses the power to give the uric acid and phenol reactions. At the end of fractionation even the last residue ceased to give these reactions, and we must assume, therefore, that the substance giving the reaction has been destroyed during the manipulation. It is quite conceivable that this substance plays an important rôle; it is present in the original crystallisation and gradually disappears during the purification.

The substance (M.P. 235°) gave the following figures on analysis; after being dried at 100° in vacuo :

3.432 mg. gave 7.345 mg. CO_2 and 1.11 mg. H_2O ; 58.37 % C and 3.62 % H
3.784 ,, 8.11 ,, ,, 1.325 ,, ; 58.45 ,, ,, 3.92 ,,
3.415 ,, 0.343 cm^3 N at 18.5° and 702 mm.; 10.87 % N
2.562 ,, 0.252 ,, ,, 15.5 $^{\circ}$,, 713 mm.; 10.92 ,,

Calc. for $\text{C}_6\text{H}_5\text{O}_2\text{N}$ (123.05): 58.3 % C, 4.08 % H, and 11.34 % N

A sample of this substance was converted into the picrate (the other two substances do not form picrates) by precipitation with a water solution of picric acid. The precipitate melted when dried at 217° (uncorr.) and was recrystallised from water, forming light yellow coloured

prisms, which melt at 219° (uncorr.). The picrate gave the following figures on analysis:

4.70 mg. gave 7.115 mg. CO_2 and 0.98 mg. H_2O ; 41.29 % C and 2.33 % H
 2.966 „ 0.436 cm^3 N at 19.5° and 708 mm.; 15.99 % N
 4.212 „ 0.61 „ „ 18° „ 711 „ ; 15.89 % N
 0.1061 gm. gave 0.1652 gm. nitronpicrate; 65.91 % picric acid
 0.1261 „ 0.1951 „ „ ; 65.49 % „

Calc. for $\text{C}_{12}\text{H}_9\text{O}_9\text{N}_4$ (352.12):

40.9 % C, 2.29 % H, 15.91 % N, and 65.05 % picric acid

This substance appears to be identical by its composition, properties and solubility with nicotinic acid, only a slight difference was observed in the melting point which was a few degrees higher. It is not unlikely that nicotinic acid is a decomposition product of the vitamine.

Investigation of rice-polishings.

The preparation of the extract was carried out conformably with the previously described method⁶, the extract from 380 kg. of rice-polishings being used as starting material. Just as in the case of yeast only the vitamine-fraction was carefully investigated. Although the investigation is not yet completed, two substances have been isolated, one of which has proved to be identical with the nicotinic acid from yeast. The initial vitamine-fraction obtained from 380 kg. of polishings amounted to 2.5 gm. This fraction was recrystallised from dilute alcohol, and a substance was obtained in the form of needles, which melted at 233° , the temperature previously found for a substance of similar origin. The yield was 1.8 gm. A second recrystallisation did not raise the melting point. The substance gives no Millon, and no uric acid and phenol reaction and is sparingly soluble in water.

The following figures were obtained on analysis:

4.796 mg. gave 10.34 mg. CO_2 and 1.685 mg. H_2O ; 58.80 % C, 3.93 % H
 4.212 „ 9.095 „ „ 1.465 „ ; 58.89 % C, 3.89 % H
 4.367 „ 0.420 cm^3 N (18° , 713 mm.); 10.58 % N
 2.692 „ 0.263 „ (18° , 706 „); 10.64 % N

These figures correspond with the formula $\text{C}_{26}\text{H}_{20}\text{O}_9\text{N}_4$ (532.24), which requires: 58.62 % C, 3.79 % H and 10.53 % N. When treated with nitrous acid in van Slyke's apparatus only a small part of the nitrogen is liberated. The nitrogen in this substance could not be estimated by Kjeldahl's method.

The mother liquid of this substance yields when slowly evaporated colourless needles identical with those obtained from yeast. The crystals

melted at 234° (uncorr.) and were very soluble in water. When mixed with the nicotinic acid from yeast no depression of melting point was observed.

On analysis the following figures were obtained:

4.217 mg.	gave	9.025 mg. CO_2	and	1.48 mg. H_2O	; 58.37 % C, 3.93 % H
4.276	"	9.16	"	1.55	" ; 58.45 % C, 4.06 % H
3.11	"	0.315 cm^3 N	(714 mm., 19.5°)	11.11 % N	
3.608	"	0.362	" (712 mm., 19.5°)	10.97 % N	

The substance was dried at 100° in vacuo. Calculated for nicotinic acid $\text{C}_6\text{H}_5\text{O}_2\text{N}$ (123.05): 58.3 % C, 4.08 % H and 11.34 % N. For further identification the substance was converted into the picrate. The same light yellow coloured prisms were obtained as before, which melted at 218° . When mixed with the picrate from yeast no depression of melting point took place:

4.471 mg.	gave	6.74 mg. CO_2	and	0.895 mg. H_2O	; 41.12 % C, 2.24 % H
2.404	"	0.349 cm^3 N	(707 mm., 19°)	15.79 % N	

These figures correspond to the picrate of nicotinic acid calc. for $\text{C}_{12}\text{H}_8\text{O}_9\text{N}_4$ (352.12): 40.9 % C, 2.29 % H, 15.91 % N. It seems at present unlikely that the nicotinic acid derives from trigonelline, which has never yet been detected in rice-polishings. From the mother liquid of nicotinic acid another substance was isolated in minute quantities which gives the Millon reaction before purification. The fractionation of rice-polishings is being continued on these lines, special attention being paid to the point whether these substances occur combined or free.

Experiments on animals.

Each fraction obtained and also the pure substances isolated during this investigation were tested on animals. The results obtained with yeast are fairly clear. The original crystalline fraction cures very well, as has been already stated in my preliminary communication. This fraction could be separated into three substances, two of which, namely the substance $\text{C}_{24}\text{H}_{19}\text{O}_9\text{N}_5$ together with nicotinic acid, give, when administered together, very satisfactory results. The substance $\text{C}_{20}\text{H}_{20}\text{O}_9\text{N}_6$ does not seem to have any effect. The results would rather seem to indicate that it inhibits somewhat the action of the two others. The experiments have been performed on pigeons, the animals being kept on polished rice throughout the experiment.

No. of animal	Substance administered	Dose	Effect	Survive
1	Crude product yeast ...	4 mg.	Cure in 3 hrs.	4 days
2	" " " ...	8 "	Cure in 2 hrs.	6 "
3	" " " ...	4 "	Cure in 3 hrs.	4 "
4	" " " ...	8 "	Cure in 3 hrs.	4 "
5	Subs. I yeast ...	8 "	Partial recovery in 7 h.	3 "
6	" " " ...	4 "	Fairly good improvmt.	4 "
7	" " " ...	2 "	Improvmt. for few hrs.	3 "
8	5 mg. 1 yeast and 2 mg. nicotinic acid	—	Cured in 2½ hrs.	4 "
9	Nicotinic acid ...	1 cg.	Improvement 5½ hrs.	2 "
10	Mixture of I, II and nic. ac.	1 "	No improvement ...	—
11	Nicotinic acid ...	5 mg.	Temporary improvmt.	3 days
12	Mixture of I and nicot. acid yeast	4 a. 2 "	Cure in 3 hrs.	5 "
13	" " " "	3 a. 2 "	Cure in 2 hrs.	6 "
14	Nicotinic acid ...	4 "	No improvement ...	2 "
15	" " " "	4 "	Temporary recovery...	1 day
16	Mixture of I and nicot. acid	4 a. 2 "	Cure in 2½ hrs.	7 days
17	" " " "	3 a. 2 "	Cure in 4 hrs.	4 "
18	Subs. II yeast ...	5 "	No effect ...	—
19	" " " "	5 "	No effect ...	1 day
20	The mixture of three ...	1 cg.	Temporary improvmt.	2 days
21	" " " "	1 "	Temporary improvmt.	1 day

CONCLUSIONS.

1. The vitamine-fraction from yeast has been separated into three substances: a substance of the formula $C_{24}H_{19}O_9N_5$, a substance of the formula $C_{23}H_{23}O_9N_5$ and what appears to be nicotinic acid (m-pyridine-carboxylic acid). The first substance mixed with nicotinic acid seems to be necessary for curing pigeons.

2. The vitamine-fraction from rice-polishings has up to the present been separated in two substances: one of the formula $C_{26}H_{20}O_9N_4$ and nicotinic acid. The results concerning their curative power will be published after the chemical investigation of all the fractions has been completed.

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IV.

Über die physiologische Bedeutung gewisser bisher unbekannter Nahrungsbestandteile, der Vitamine.

Von

Casimir Funk, London.

Mit 6 Abbildungen im Text und 3 Abbildungen auf Tafel I.

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Einleitung.

Die Forschungen der letzten Zeit über Beriberi haben neue Tatsachen ans Licht gebracht. Es wurde nämlich experimentell und klinisch erwiesen, dass sich in der Nahrung besondere, bisher unbekannte, lebenswichtige Substanzen befinden. Fehlen diese Stoffe — was infolge einer fehlerhaften Zusammensetzung der Nahrung geschehen kann — so entstehen Massenerkrankungen von scharf ausgeprägtem Typus. Diese Ergebnisse der neuen Forschung sind von eminenter praktischer Wichtigkeit: sie erklären uns das Wesen geheimnisvoller Volkskrankheiten und ermöglichen demnach ihre erfolgreiche Prophylaxe und Heilung. Wir erleben es voraussichtlich, dass jene Krankheiten gänzlich besiegt werden. Es ist andererseits für die Physiologie eine ganz neue Tatsache, dass durch Fehlen gewisser Substanzen in der Nahrung, manche mit konstanten Symptomen verlaufende Krankheiten und sogar der Tod herbeigeführt werden kann, obwohl es in der Nahrung weder an Kalorien, noch an Stickstoff und Salzen mangelt. Diese neuen Tatsachen müssen unsere Anschauungen über die Stoffwechsellehre bedeutend modifizieren.

Die Krankheiten, die durch die obige Ursache charakterisiert sind, wollen wir in dieser Zusammenfassung als partielle Unterernährungskrankheiten (Deficiency diseases) bezeichnen. Dass es sich hier wirklich nur um partielle Unterernährung handelt, kann dadurch bewiesen werden, dass — bei gleichbleibender Nahrung — Genesung herbeigeführt werden kann, wenn der fehlende Stoff wieder zugeführt wird.

Zu diesen Krankheiten wollen wir vorläufig Beriberi, Skorbut, Barlowsche Krankheit, Schiff-Beriberi und Geflügel-Polyneuritis rechnen, doch werden sich im Verlaufe unserer Ausführungen Gesichtspunkte finden, die es wahrscheinlich machen werden, dass die Gruppe dieser Krankheiten noch erweitert werden kann. Ich habe für die hier in Betracht kommenden Stoffe den Namen Vitamine vorgeschlagen.

Beriberi.

Von den partiellen Unterernährungskrankheiten ist besonders sorgfältig die sog. Beriberi (japan. Kakke) studiert worden, und deshalb wollen wir am längsten bei ihr verweilen. Die Krankheit, die seit etwa 20 Jahren sehr intensiv das Interesse von vielen Forschern in Anspruch genommen hat, kommt fast ausschliesslich in Ländern vor, deren Hauptnahrung Reis bildet. Nur ganz ausnahmsweise ist in letzter Zeit berichtet worden (Little 1), dass auch der Genuss von weissem Brot wie z. B. in New Foundland die Ursache von Beriberi sein kann. Ganz besonders verbreitet ist die Krankheit in Japan, auf der malaiischen Halbinsel und auf den Philippinen, und das sind auch die Länder, wo dem Studium dieser Krankheit besondere Aufmerksamkeit

gewidmet wurde. Über das Wesen der Krankheit mit seinen noch jetzt zum Teil rätselhaften Symptomen blieben wir lange Zeit im Dunkeln. Es ist wohl zuerst als eine Art von Intoxikation oder als Infektion aufgefasst worden, und nur langsam hat sich hier die richtige Erkenntnis den Weg gebahnt.

Es ist wohl zuerst von Wernich (2) und van Leent (3) vermutet worden, dass zwischen dem Reiskonsum und Beriberi ein kausaler Zusammenhang existiere. Takaki (4) gelang es dann, durch Zusatz von Fleisch in der japanischen Marine die Krankheit sehr bedeutend einzuschränken. Viel wichtigere Fortschritte wurden dagegen auf Java von holländischen Forschern erzielt. Auf Grund von sehr reichem statistischem Material, das in 101 japanischen Gefängnissen in den Jahren 1895—1896 gesammelt wurde, gelang es Vordermann (5) nachzuweisen, dass die Krankheit mit dauerndem Konsum von weissem (poliertem) Reis in Beziehung gebracht werden kann. Diese Tatsache wurde an sehr grossem Material von Braddon (6) auf der malaisischen Halbinsel bestätigt. Er konnte zeigen, dass die Eingeborenen, wie die Stämme von Tamilen, welche sich von Reis nähren, der als „cured“ oder als „parboiled“ bezeichnet wird, frei von dieser Krankheit sind. Dieser Reis zeichnet sich dadurch aus, dass er vor dem Gebrauch gedämpft wird, wodurch die Spelzen leicht abgehen und das Polieren unnötig wird.

Im Jahre 1897 fand Eykman (7), dass Beriberi durch einen dauernden Konsum von weissem (poliertem) Reis verursacht ist, d. h. dass durch den Polierprozess ein Bestandteil des Reises verloren geht, der vor dem Ausbruch der Krankheit schützt. Als der wichtigste Teil des Reiskorns wurde von den holländischen Autoren das sog. „Silberhäutchen“ (silvervlissen) betrachtet, das die aus dem weissen Reis entstehenden Toxine neutralisieren sollte.

Daraufhin gelang es Fraser und Stanton (8) an der Hand von Reisschnitten klarzumachen, dass die schützende Substanz auf der Peripherie des Reiskornes lokalisiert ist. Es liess sich auf diese Weise zeigen, dass beim Polieren des Reises Substanzverluste eintreten, die beim unpolierten und gedämpften Reis niemals vorkommen. Zum klaren Verständnis dieser Unterschiede bringen wir die Original-Zeichnungen der genannten Autoren, an denen die obengenannten Unterschiede ohne weiteres zu erkennen sind. (Vgl. die Tafel.)

Was die Krankheit selbst anbelangt, so existieren verschiedene Typen. In manchen Fällen magern die Patienten stark ab, in anderen zeigen sie ausgebreitete Ödeme. Doch die charakteristischsten Erscheinungen sind die Nervensymptome, die sich in Lähmungen, Muskelatrophien und Kontrakturen der Extremitäten äussern. In akuten Fällen tritt Tod durch Herzschwäche ein. Pathologisch-anatomische Veränderungen werden hauptsächlich an den peripheren Nerven gefunden, und zwar alle Stadien der Entartung, nicht nur an den Extremitäten, sondern auch am Vagus und Phrenicus, einigen Hirn-

nerven, ferner am Sympathicus und im Rückenmark. Die kranken Muskeln sind entartet und atrophiert, entsprechend den degenerierten Nervenendigungen. Oft wurde Hydropericardium gefunden (Entartung der Herzbeutelnerven). Das Herz, besonders das rechte, ist stark dilatiert, der Herzmuskel fettig entartet. Obwohl behauptet worden ist, dass manchmal spontane Heilungen vorkommen, so scheint dies von unserem Standpunkt aus unwahrscheinlich, wenn keine Änderung in der Diät vorgenommen wird.

Trotz der Erkenntnis, dass Beriberi mit der Reismahrung im Zusammenhang steht, wären wir nicht sehr weit vorgeschritten, wenn es nicht Eykman (9) gelungen wäre durch Verfütterung von Reis an Geflügel (Hühner, Enten, Gänse und Tauben) eine Krankheit hervorzurufen, die er *Polyneuritis gallinarum* nennt (s. Fig. 1, 2 u. 3, die dem Werke Fraser und Stanton entnommen



Figur 1. Polyneuritis. 1. Tag der Krankheit.

sind). Die Symptome dieser Krankheit sind folgende: Die Vögel, die am Anfang weissen Reis sehr gern fressen, verlieren den Appetit vollständig, so dass sie schliesslich nur künstlich ernährt werden können. Sie zeigen sehr oft grosse Gewichtsabnahme, die manchmal bis auf 45 % steigen kann. Nach einiger Zeit, die übrigens sehr schwankt (1 bis 6 Wochen), bekommen sie die akuten Symptome, die in einer Lähmung der Flügel und Beine bestehen. In den meisten Fällen findet man den Kropf prall mit Reis gefüllt. Eine sehr eigenartige Erscheinung bei den kranken Tieren ist die Kopfstellung, durch eine spastische Kontraktur der Halsmuskeln verursacht, der Kopf wird nämlich gegen den Rücken oder gegen den Bauch gepresst. (Vgl. die beig. Photographie.) Sich selbst überlassen, leben die Tiere kaum 12–24 Stunden.

Man fand Eykman, dass die Tiere nur dann krank werden, wenn man sie mit weissen Reis füttert, dem durch Polieren das Perikarp entzogen wird. Beim Verabreichen von Paddy (unpoliertem Reis) oder von gedämpftem



Figur 2. Polyneuritis. 1. Tag der Krankheit.



Figur 3. Polyneuritis. 2. Tag der Krankheit.

Reis (cured rice) bleiben die Vögel gesund. Somit wurde die volle Analogie mit der menschlichen Beriberi erwiesen. Wie wir später sehen werden, geht



Figur 4.

Fettige Degeneration des N. Ischiadicus eines an Polyneuritis erkrankten Huhnes.

die Analogie so weit, dass wir ohne weiteres berechtigt sind, Schlüsse von einer Krankheit auf die andere voll zu übertragen. Pathologisch-anatomisch sind Beriberi und Polyneuritis gallinarum vollständig identisch (s. Fig. 4.)

Somit war uns ein Mittel an die Hand gegeben die Untersuchungen weiter zu führen und etwaige Hypothesen experimentell zu prüfen. Dies ist dann gleich darauf in Angriff genommen worden. Was die hydropische Form der Beriberi (Epidemie dropsy) betrifft, so hat Greig (10) gezeigt, dass sie ebenfalls durch weissen Reis verursacht wird. Diese Form wird deshalb zusammen mit Beriberi besprochen.

Das Wesen der Krankheit und die chemische Natur der heilenden Substanz.

Auch hier war es Eykman (l. c. 9), der die ersten Schritte in dieser Richtung machte. Er fand nämlich, dass der Zusatz von Perikarp oder Reiskleie zum weissen Reis den Ausbruch der Krankheit bei den Tieren zu verhüten instande ist. Für diese Tatsache fand er allerdings nicht die richtige Erklärung, er glaubte nämlich, dass sich aus der Stärke des Kornes Toxine entwickeln, die auf das Nervensystem deletär wirken, und die durch den Zusatz von Perikarp verhindert werden. Es ist ja weiter nicht merkwürdig, da in damaligen Zeiten das Wesen der Krankheit recht rätselhaft war.

Er machte die wichtige Beobachtung (11), dass der wässrige Extrakt der Reiskleie heilende Eigenschaften besitzt. Phytin wurde darin entdeckt und gezeigt, dass es unwirksam ist. Die heilende Substanz ist dialysierbar und fällt durch Zusatz von Alkohol nicht aus. Die im Jahre 1897 gemachten Beobachtungen waren übrigens die einzigen, auf die sich die moderne Beriberiforschung stützen konnte. Es gebührt daher Eykman das Verdienst, die Grundlagen für

in dieser Zusammenfassung aufgeführten Untersuchungen geschaffen zu haben.

Die Untersuchungen wurden daraufhin auch von Gryn's (12) aufgenommen. Er konnte die Angaben von Eykman völlig bestätigen. Er war übrigens der erste Forscher, der sich klar für die auch jetzt gültige Auffassung der Beriberi ausgesprochen hat. Er sagte nämlich, dass die Krankheit ausbricht, wenn in der Nahrung Stoffe fehlen, die für den Stoffwechsel des peripheren Nervensystems von Bedeutung sind. Ähnliche heilende Stoffe wie in der Reiskleie konnte er in einer Bohnenart, in Katjang-idjoe (*Phaseolus radiatus*) und in Fleisch nachweisen, und konnte zeigen, dass die Nahrungsmittel ihre heilende Kraft verlieren, wenn sie auf 120° C erhitzt werden. Diese Experimente waren von grosser Bedeutung für die weiteren Untersuchungen, sie wurden auch von Eykman (l. c. 11) bestätigt. Bréaudat (13) benutzte Reiskleie in Fällen von menschlicher Beriberi mit gutem Erfolge. Fraser und Stanton (14) versuchten die Substanz aus der Reiskleie näher zu charakterisieren. Sie fanden, dass dieselbe in starkem Alkohol löslich ist, und nach der Eliminierung von alkohollöslichen Proteinen ihre Aktivität behält. Sie führten Analysen von vielen Reissorten aus und machten die Beobachtung, dass Reis, der arm an Phosphor ist, den Ausbruch von Beriberi auszulösen vermag. Daraufhin empfahlen sie den Gehalt an Phosphor als einen praktischen Indikator, der besagt, ob der Reis schädlich oder unschädlich ist. So sollten z. B. Reise, die mindestens 0,46 % P_2O_5 enthalten, als unschädlich betrachtet werden. Doch haben Schüffner und Kuenen (15) klar bewiesen, dass die Zubereitung der Reissnahrung ebenfalls wichtig ist. Es muss nämlich der Reis mitsamt der Brühe genossen werden. Wird die Brühe regelmässig weggegossen, so kann leicht Beriberi entstehen. Es ist wohl möglich, dass diese empirisch gefundene Tatsache von praktischem Wert ist. Auf diese Zahlen gestützt betrachtete Schaumann (16) Beriberi als eine durch Mangel an organisch-gebundenem Phosphor verursachte Störung des Stoffwechsels. Diese Theorie wurde daraufhin von Schaumann auf andere partielle Unterernährungskrankheiten, wie auf Skorbut und Schiffberiberi, erweitert. Sie fand Anhänger (Simpson und Edie [17]) und beherrschte dieses Gebiet der Pathologie die letzten Jahre hindurch. Zu jener Zeit war wohl diese Hypothese am plausibelsten wegen des grossen Unterschieds im Phosphorgehalt von weissem Reis gegenüber der Reiskleie. Wir werden noch weiter unten auf dieselbe zurückkommen.

In den letzten Jahren erschien in rascher Folge eine ganze Anzahl von Arbeiten, die sich mit der chemischen Natur der heilenden Substanz befassten. Zu ihrer Darstellung wurden verschiedene Nahrungsmittel verwendet. Hulshoff Pol (18) konnte nachweisen, dass ein wässriger Extrakt von Katjang-idjoe-Bohnen die heilende Kraft behält, nachdem man die Lösung mit Bleiacetat gereinigt hat. Aus dem in dieser Weise gereinigten

Filtrat erhielt dieser Autor eine krystallinische Substanz, die er X-Säure nennt; ob die Substanz irgendwelche physiologische Eigenschaften aufweist, darüber fehlen nähere Angaben.

Schaumann (19), der die Reihe der heilenden Nahrungsstoffe um einen neuen bereicherte, nämlich Hefe (worauf Thomson und Simpson [20] dieselbe zur Behandlung der Beriberi anwandten), versuchte die Wirkung der schon bekannten Hefebestandteile. Unter anderem versuchte er die Wirkung von Hefenukleinsäure und Hefelecithin, ohne eindeutige Resultate erhalten zu haben. Den Grund dafür werden wir später sehen. Eykman (21) konnte zeigen, dass man den aktiven Bestandteil der Hefe mit Alkohol extrahieren kann (88%). Verfasser (22) konnte schon früher diese Beobachtung machen, doch gelingt es in dieser Weise nur einen Teil des aktiven Prinzips zu extrahieren. Sogar nach vielstündigem Extrahieren mit Alkohol, unter häufigem Wechsel desselben, gelang es nur einen geringen Teil der Substanz aus der Hefe zu gewinnen. Die Hauptmasse war in der Hefe zurückgeblieben und konnte auf keine Weise isoliert werden. Teruuchi (23) extrahierte Reiskleie mit schwacher Salzsäure, neutralisierte die Lösung, wobei Phytin ausfiel, dann wurde das Filtrat eingedampft und der Rückstand mit Alkohol extrahiert, die so erhaltene Lösung erwies sich als aktiv und enthielt nur einen geringen Teil des ursprünglichen P-Gehalts. Ähnliche Resultate wurden auch von Chamberlain und Vedder (24) erhalten. Sie fanden, dass die heilende Substanz durch Tierkohle absorbiert wird. Sie wollten darauf ein Darstellungsverfahren gründen, das aber fehlschlug. Shiga und Kusama (25) fanden, dass das aktive Prinzip der Reiskleie durch Erhitzen auf 130° C in 1/2%iger Salzsäurelösung, oder in 1%iger Sodalösung vernichtet wird, nicht aber bei 100° C.

Darstellungsversuche aus der Reiskleie wurden auch von Tsuzuki (26) mit wenig Erfolg versucht. Wegen des enormen Reichtums der Reiskleie an Phytin, wurde diese Substanz von Aron und Hocson (27) vorübergehend als die heilende betrachtet; die guten Resultate, die von diesen Autoren erhalten wurden, können vielleicht dadurch erklärt werden, dass das Phytin etwas von der wirksamen Substanz mitgerissen hatte. Die Experimente mit Phytin wurden schon früher von Eykman ausgeführt und als resultatlos bezeichnet; sie konnten auch von Cooper und Casimir Funk (28) widerlegt werden. Kilbourne (29) betrachtete den Mangel an Kaliumsalzen als die Ursache der Krankheit, ohne jedoch experimentelle Beweise für seine Anschauung zu liefern.

Es darf hier nicht übergangen werden, dass trotz der grossen Anzahl der Beweise, die die Beriberi-Krankheit ohne den geringsten Zweifel als eine partielle Unterernährungskrankheit klassifizieren, Kohlbrugge (30) dieselbe als eine Gärungskrankheit bezeichnete. Er isolierte aus dem Reis eine Bakterienart, die er *B. oryzae* nennt, die bei Hühnern Polyneuritis in drei

Fagen auslösen soll. Der schwache Punkt dieser Theorie ist sein Versuch, die heilende Wirkung der Reiskleie zu erklären. *B. oryzae* solle eine Gärung von einer speziellen Art hervorrufen, die durch die aus der Reiskleie entstehende Säure aufgehoben wird. Das epidemische Auftreten von Beriberi legte vielen Autoren den Gedanken nahe, dass es sich hier um eine Infektionskrankheit handelt. Es wurde lange Zeit übersehen, dass es sich in diesen Fällen um Personen handelte, die unter identischen Bedingungen lebten, in Gefängnissen, Kasernen, und deren Diät annähernd die gleiche war. Es wurde ebenfalls oft behauptet, dass beim Transport von Kranken von einem Pavillon in einen anderen die Krankheit plötzlich zum Stillstand gebracht wurde. In diesen Fällen zeigte eine eingehende Untersuchung, dass der Transport entweder eine Änderung in der Diät, oder eine Änderung in den Kochapparaten zur Folge hatte. Diese Tatsachen müssen um so mehr betont werden, da es immer noch Anhänger der Infektionstheorie gibt.

Wollen wir unsere Kenntnisse über die chemische Natur des aktiven Prinzips bis 1911 kurz resümieren, so waren folgende Tatsachen mit Sicherheit bewiesen:

1. Die Substanz ist löslich in Wasser, Alkohol und säurehaltigem Alkohol.
2. Die Substanz ist dialysierbar.
3. Durch Erhitzen auf 100°C wird sie zerstört.
4. Sie gehört weder zu den Salzen, noch zu den Proteinen.

Obwohl nicht viel über die chemische Natur der heilenden Substanz bekannt war, war es wohl an der Zeit diese Substanz mit rein chemischen Methoden zu untersuchen. Die Frage wurde deshalb von physiologischen Chemikern in Angriff genommen, und hat Resultate gegeben, die im nächsten Abschnitt zusammengefasst werden.

Die Untersuchung der chemischen Natur der heilenden Substanz mit physiologisch-chemischen Methoden.

Die Untersuchungen hatten, wie wir gesehen haben, ergeben, dass die Substanz sehr instabil ist. Es war daher zu befürchten, dass dieselbe den chemischen Eingriffen überhaupt nicht widerstehen würde. Als ich die Beriberi-Frage in der Mitte des Jahres 1911 aufnahm, ergaben sich vor allem drei Fragestellungen, nämlich: ist die Substanz stabil genug, um den chemischen Manipulationen zu widerstehen, zu welcher Klasse der chemischen Körper muss sie gerechnet werden, besitzt sie einfachen Bau, d. h. ist sie gebunden oder frei in der Natur vorhanden, und ist sie in dieser letzten Form wirksam?

Um diese Fragen zu entscheiden unternahm der Verfasser (l. c. 28) eine Reihe von Untersuchungen, indem trockene Presshefe 24 Stunden mit 20% H_2SO_4 hydrolysiert wurde. Das neutralisierte Hydrolysat hat sich als aktiv

erwiesen. Diese merkwürdige Stabilität gegenüber Säuren machte es sehr wahrscheinlich, dass die in Betracht kommende Substanz stickstoffhaltig ist, und durch die dieser Klasse üblichen Eigenschaften gekennzeichnet ist. Auf diese Ideen gestützt konnte dann eine systematische Untersuchung der Reiskleie in Angriff genommen werden. (Casimir Funk [31].)

Untersuchung der Reiskleie.

Da keine chemische Reaktion für die wirksame Substanz zu jener Zeit bekannt war, wurde jede erhaltene Fraktion an Tauben, die an Polyneuritis litten, geprüft. Als der richtige Moment für die Einführung (die übrigens zuerst per os geschah) wurde das Erscheinen von Nacken-, Flügel- und Bein- kontrakturen angesehen. Sich selbst überlassen, sterben die Vögel nach etwa 12 Stunden, vom Anfang dieses Stadiums gerechnet.

Ganze Reihen von Vorversuchen wurden ausgeführt um eine möglichst einfach zusammengesetzte wirksame Lösung zu erhalten. Dies wurde erreicht, indem die Reiskleie, die der Hauptsache nach aus Cellulose, Phytin und Fett zusammengesetzt ist, durch Schütteln auf der Schüttelmaschine mit Alkohol, der zu einem gewissen Grade mit gasförmiger Salzsäure gesättigt war, extrahiert wurde. Dieses Verfahren besass gegenüber den älteren den Vorzug, dass die Lösung von alkohol-unlöslichen Substanzen vollkommen frei wurde. Die alkoholische Lösung wurde daraufhin im Vakuum eingedampft, wobei schliesslich ein fettiger Rückstand zurückblieb. Der Rückstand wurde auf dem Wasserbade geschmolzen und heiss mit Wasser extrahiert und die beiden Schichten heiss im Scheidetrichter getrennt. Der wässrige Extrakt, der sehr aktiv war, wurde mit Schwefelsäure versetzt, bis die Lösung 5%ig war, und mit einer 50%igen Phosphorwolframsäurelösung, solange noch eine Fällung eintrat, gefällt. Der erhaltene Niederschlag wurde in üblicher Weise mit Baryt zersetzt und das erhaltene Filtrat nach Eliminierung des überschüssigen Baryts auf das Heilungsvermögen gegenüber den Polyneuritis-Tauben geprüft. Die Lösung erwies sich als sehr wirksam und war phosphor-, protein- und kohlehydratfrei (l. c. 31). Die erste Schwierigkeit, der hier begegnet wurde, war die, dass es nicht möglich war, die richtige Dosis für kranke Tauben zu finden. Wie sich später herausstellte, war diese Lösung sehr reich an freiem Cholin, das für Tauben sehr giftig ist. Diese Schwierigkeit konnte überwunden werden, indem die Dosis auf die verwendete Reiskleie, die übrigens von der malaiischen Halbinsel stammte, umgerechnet wurde. Mit jeder weiteren Fraktion musste natürlich, den eingetretenen Verlusten entsprechend, die Dosis vergrössert werden.

Die Tatsache, dass die Lösung phosphorfrei war, gab der Schaumannschen Phosphormangel-Hypothese den letzten Stoss, denn hier wurde zum erstenmal deutlich gezeigt, dass eine vollkommen phosphorfreie Lösung

physiol. Bedeutung. Dieser Silber-alkalischer Ammonium-Niederschlag, der in Wasser
wirksam sein kann. Da das phosphorwolframsaure Filtrat gänzlich
inaktiv war, so musste die ganze wirksame Substanz in den Niederschlag
übergegangen sein.

Hier wurden dann die üblichen Methoden angewandt, die für die Auf-
arbeitung solcher Niederschläge gewöhnlich gute Dienste leisten. Die er-
haltene Lösung, die viel Kali enthielt und deswegen ziemlich stark
alkalisch reagierte, wurde mit Salzsäure neutralisiert und zum Sirup im
Vakuum eingedampft. Der Rückstand wurde in Alkohol aufgelöst, wobei
der grösste Teil des Kaliumchlorids zurückblieb. Die alkoholische Lösung
wurde daraufhin mit Sublimat gefällt, der erhaltene Niederschlag aus Wasser
unter Zusatz von etwas Sublimat umkrystallisiert. Die erhaltenen Krystalle
wurden in Wasser suspendiert und mit Schwefelwasserstoff zersetzt. Diese
Lösung, sowie die, die durch Eliminierung von überschüssigem Quecksilber
erhalten wurde, erwiesen sich beide als aktiv. (Das Filtrat jedoch viel
stärker). Somit gelang es nicht, eine Trennung durch Sublimat zu bewirken.
Die aus dem Niederschlage erhaltene Lösung wurde im Vakuum eingeeengt,
der Rückstand in Alkohol gelöst und mit einer alkoholischen Platinchlorid-
Lösung fraktioniert gefällt. Es wurde eine ganze Anzahl von Fraktionen
erhalten, die nach den Analysen aus reinem Cholin bestanden. Die
alkoholische Lösung war immer noch wirksam, nachdem das ganze Cholin
quantitativ entfernt worden war. Es bestand daher kein Zweifel, dass
die heilende Substanz nicht der Cholingruppe angehört. In der nach der
Fällung mit Sublimat zurückgebliebenen Mutterlauge wurden darauf verschie-
dene Fällungsmittel probiert, die die wirksame Substanz niederzuschlagen ver-
mochten.

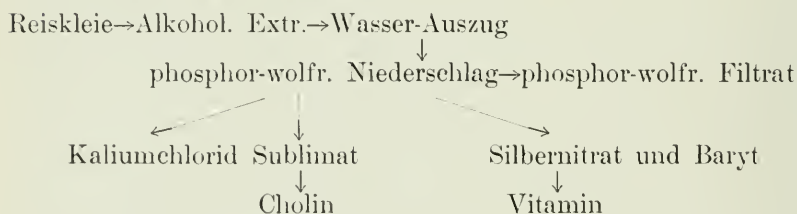
Als solches wurde Silbernitrat vom Verfasser gewählt, und zwar nur in
alkalischer Lösung, also der Histidingruppe entsprechend. Zu diesem Zweck
wurde die Lösung mit Silbersulfat von Chlor befreit. Nach Entfernung des
Silbers und der Schwefelsäure, wurde die Lösung mit Salpetersäure und
dann mit Silbernitrat so lange versetzt, bis die Lösung mit Baryt eine braune
Fällung gab.

Dann wurde eine gesättigte Barytlösung zugesetzt, solange ein Tropfen
der klaren Lösung mit ammoniakalischer Silbernitratlösung versetzt nur eine
geringe weisse Fällung ergab. Der erhaltene Niederschlag wurde von Salpeter-
säure gut ausgewaschen, mit Schwefelwasserstoff zersetzt, von letzten Spuren
Baryt mit einer sehr verdünnten Schwefelsäure befreit, und im Vakuum ein-
geengt. Die so erhaltene Lösung erwies sich als sehr aktiv. Durch lang-
sames Verdunsten der Lösung im Exsikkator unter Zusatz von Alkohol wurde
eine farblose krystallinische Substanz erhalten. Die Ausbeute betrug 0.4 g
aus 50 kg Reiskleie. Die sehr geringe Ausbeute erschwerte die weitere
Untersuchung ungemein. Es gelang trotzdem eine ganze Anzahl von Reak-
tionen auszuführen, die ergaben, dass hier offenbar eine neue Verbindung

vorlag. Die in Nadeln krystallisierte Substanz schmolz sehr stark bei 233° (unkorr.), war ziemlich schwer löslich in kaltem Wasser und in kaltem Alkohol. Die an einer geringen Substanzmenge ausgeführten Analysen führten zu der Formel $C_{17}H_{20}N_2O_7$. Der ungesättigte Charakter dieser Substanz könnte deren Instabilität gut erklären. Die Substanz, einigen kranken Tauben per os eingeführt, heilte dieselben in kurzer Zeit (3 bis 6 Stunden) vollständig. Auf Reismahrung gesetzt zeigten die Tiere erst Symptome nach etwa 10 Tagen. Es war ausserordentlich interessant zu beobachten, wie die Lähmungen allmählich wichen, der Appetit wiederkam und der Durchfall, der ebenfalls zu den charakteristischen Symptomen der Polyneuritis bei Tieren gehört, plötzlich aufhörte.

In einer späteren Arbeit (32) habe ich vorläufig den Namen Beriberi-Vitamin für die wirksame Substanz vorgeschlagen, um auszudrücken, dass es sich hier um eine stickstoffhaltige und für das Leben unentbehrliche Substanz handelt.

Um den Darstellungsgang zu veranschaulichen, wollen wir denselben in einem Schema rekapitulieren:



In einer späteren Arbeit versuchte ich das Verfahren zu vereinfachen (l. c. 32). In erster Linie wurde die Reiskleie statt mit salzsäurehaltigem, mit gewöhnlichem Alkohol extrahiert. Der wässrige Auszug wurde dann, statt mit Phosphorwolframsäure, gleich mit Silber und Baryt gefällt. Ich konnte nun die Beobachtung machen, dass dieses Extrakt sich ganz verschieden von dem früheren verhielt; er schien weniger Kali zu enthalten, auch kein freies Cholin, die Erklärung dafür werden wir später sehen. Durch Zersetzen des Silberniederschlags wurde zu meinem grossen Erstaunen nicht etwa Vitamin, sondern eine ähnliche Substanz erhalten, die bei 231° C schmolz und mit Allantoin identifiziert werden konnte. In einer Mitteilung polemischen Inhalts erhebt Schaumann (Zu dem Problem der Beriberiätiologie. Arch. f. Schiffs- und Tropenhyg. 56, 825. 1912) gewisse gegen mich gerichtete Angriffe und Prioritätsansprüche, die total unberechtigt sind. So glaubt er auch im Jahre 1911 eine N-haltige Substanz aus der Reiskleie isoliert zu haben. Diese Substanz wurde nicht weiter untersucht, weder chemisch noch therapeutisch, und die von Schaumann angewandte Methode konnte nur zur Isolierung von Allantoin führen. Die physiologische Bedeutung des Allantoins wird in einem späteren Kapitel diskutiert werden. Somit gelang es mir in

der Reiskleie drei stickstoffhaltige Substanzen zu isolieren, nämlich Vitamin, Allantoin und Cholin.

Nachdem meine Arbeit erschienen war, unternahm Schaumann (33) eine Untersuchung der Reiskleie unter Anwendung der von mir beschriebenen Methode. Er konnte eine geringe Menge einer krystallisierenden Substanz aus der Sublimatfraktion der Reiskleie darstellen und dieselbe als wirksam erkennen. Er konnte meine Resultate vollständig bestätigen.

Vor kurzer Zeit erschien eine Arbeit von Suzuki, Shimamura und Odake (34), die auch dasselbe Thema behandelt. Die angewandte Methodik weicht nur in Kleinigkeiten von der meinigen ab. Sie extrahierten entfettete Reiskleie am Rückflusskühler mit Alkohol drei Stunden einige Male bis die Extraktion vollständig war. Die alkoholischen Auszüge wurden vereinigt und eingedampft. Der Extrakt wurde mit Wasser verdünnt, mit Schwefelsäure versetzt (bis die Lösung 3prozentig war) und mit 30 proz. Phosphorwolframsäurelösung gefällt. Zur Extraktion der Reiskleie wurde keine Salzsäure angewandt, wodurch die etwas abweichenden Resultate später ihre Erklärung finden werden. Die durch Zersetzung des Phosphorwolframsäureniederschlages erhaltene Lösung erwies sich als sehr aktiv und wurde Rohoryzanin I genannt. Die Ausbeute betrug 1,2 g aus 300 g entfetteter Reiskleie. Diese Fraktion heilte Tauben in Mengen von 3—4 cg prompt. Das Filtrat der Phosphorwolframsäurefällung erwies sich dagegen als unwirksam. Die Fraktion war löslich in Wasser und in Alkohol, schwach sauer, zeigte eine positive Millon- und Diazoreaktion, Reaktionen, die in meinem Falle negativ waren. Die Substanz war durch Bleiessig teilweise fällbar, besser durch Zusatz von Ammoniak, durch Sublimat, Quecksilberacetat und -nitrat wird die Substanz partiell gefällt. Die Autoren führten daraufhin eine Aufspaltung dieser Fraktion aus, wobei sie bemerken, dass dabei die physiologische Wirkung vollständig verloren gehen soll. Wie wir später sehen werden, muss dies als irrtümlich bezeichnet werden, auch wurden die diesbezüglichen Tierexperimente nicht aufgeführt. Aus meinen Hefeexperimenten ergibt sich, dass die Substanz gegen Säuren höchst resistent ist. Es ist wohl möglich, dass diese Angabe der japanischen Autoren aus den Experimenten Gryns (l. c. 12) ohne weitere Versuche abgeleitet ist. Dagegen scheint das Vitamin gegen Alkali wenigstens nach meinen Experimenten sehr empfindlich zu sein.

Die Spaltung wurde durch zweistündiges Erhitzen mit 3%iger Salzsäure bewerkstelligt. Beim Erkaltenlassen schieden sich aus der Lösung gelbbraun gefärbte Krystalle ab, die aus heissem Alkohol umkrystallisiert wurden. In dieser Weise wurden zweierlei Substanzen erhalten, von denen die erste bedeutend schwerer löslich ist als die zweite. Beide Substanzen sollen sehr schwer im Wasser löslich sein, sauer reagieren; sie sind in Alkali löslich und werden durch Säuren wieder aus der Lösung abgeschieden. Bei der Analyse gab die erste Substanz die Formel $C_{18}H_{16}N_2O_9$ und wurde α -Säure genannt,

die zweite Substanz besass die Formel $C_{10}H_8NO_4$ und wurde β -Säure genannt. Beide Substanzen sollen angeblich die Millonische und die Diazoreaktion geben. Leider fehlen hier die Schmelzpunkte und die Tierexperimente, also das Entscheidende. Es scheint mir nicht unwahrscheinlich, dass die α -Säure vielleicht identisch mit dem von mir beschriebenen Vitamin ist. Aus dem Hydrolysat konnte ausserdem Cholin und Nikotinsäure (Pyridin-m-carbonsäure, Schmelzpunkt 214°) in Form von Pikraten isoliert werden, ferner Traubenzucker. 100 Teile Rohoryzanin gaben 10 Teile α - und β -Säure, 30 Teile Cholin und Nikotinsäure und 23 Teile Glukose.

1 g Rohoryzanin gab 0,044 g N.

0,055 durch Phosphorwolframsäure fällbar

0,009 in anderer Form (α - und β -Säure).

Wie diese Zusammensetzung zeigt, ergibt die von den japanischen Autoren untersuchte Reiskleie wesentlich abweichende Resultate. Dies ist vielleicht zum Teil durch die Nichtanwendung von Salzsäure zu erklären, wodurch keine sekundäre Spaltung zu befürchten war. Andererseits scheint die japanische Reiskleie anders zusammengesetzt zu sein (Gegenwart von Nikotinsäure). Die von mir benutzte Reiskleie stammte von der malaiischen Halbinsel und konnte ausserdem durch langdauernde Lagerung eine wesentliche Spaltung erlitten haben.

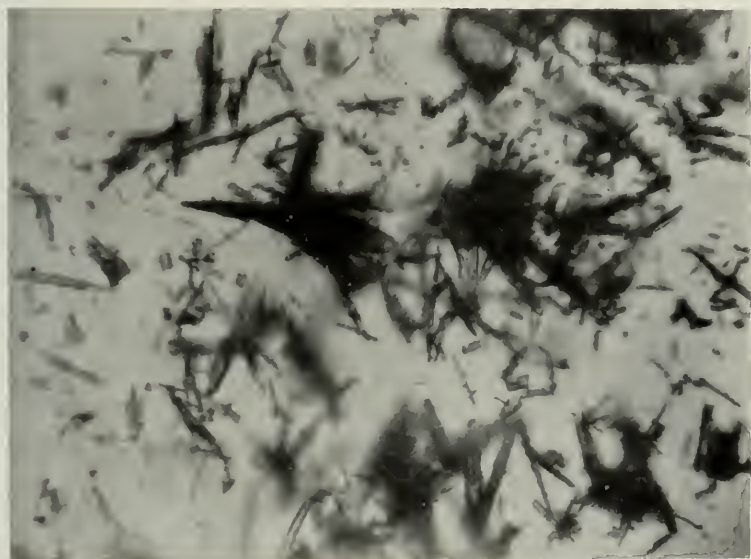
Das Rohoryzanin I wurde daraufhin einer weiteren Reinigung unterworfen. es wurde in Wasser gelöst und mit einer 20%igen Tanninlösung so lange gefällt, bis nur eine schwache Trübung entstand. Die Fällung wurde abfiltriert, mit einer 1%igen Tanninlösung rasch gewaschen. Der Niederschlag wurde in einem Mörser einige Male mit einer 3%igen Schwefelsäure verrieben, bis vollständige Lösung eintrat. Die Lösung wurde mit einem Überschuss von Baryt versetzt, die Fällung filtriert und im Filtrat der Überschuss an Baryt mit Schwefelsäure entfernt. Die Lösung wurde mit Äther geschüttelt und eingedampft. In dieser Weise wurde ein Präparat erhalten, das Rohoryzanin II genannt wurde, und das dreimal wirksamer war wie das Präparat I.

Wurde eine konzentrierte wässrige Lösung dieses Präparats mit einer wässrigen Pikrinsäurelösung unter Vermeidung eines Überschusses verrieben, so schied sich eine flockige Fällung ab, die beim Stehen in der Kälte krystallinisch wurde. Die Krystalle enthielten noch etwas Nikotinsäurepikrat beigemengt, beim vorsichtigen Arbeiten bleibt letzteres in Lösung. Das Oryzanin-pikrat wurde durch Lösen in kaltem Aceton und Verdunstenlassen der Lösung im Exikkator umkrystallisiert: gelbbraune, sternförmig gruppierte mikroskopische Nadeln. Den Tauben verabreicht erwies sich das Pikrat als sehr aktiv, eine Menge, die 2 cg Pikrat entsprach, entfaltete auf die Tiere eine ausserordentlich günstige Wirkung. Die reine Substanz wurde nur zwei Tauben verabreicht. Die Menge des erhaltenen Pikrats war so gering, dass es sogar

für eine Schmelzpunktsbestimmung nicht ausreichte. Die Frage, ob das reine Oryzanin dieselben Spaltungsprodukte wie das Rohoryzanin I geben würde, wurde deshalb von den Autoren offen gelassen. Inzwischen wurde von mir die Methode von Suzuki genau nach den Angaben der Autoren einer Nachprüfung unterzogen, die bis jetzt vollständig resultatlos verlief. Die Gründe dafür wurden schon oben diskutiert. Ich gehe jetzt zu meinen Untersuchungen der Hefe über.

Untersuchung der Hefe.

Die Hefe wurde von mir nach zwei verschiedenen Methoden untersucht (l. c. 32). Nach den erhaltenen Resultaten unterliegt es wohl keinem Zweifel,

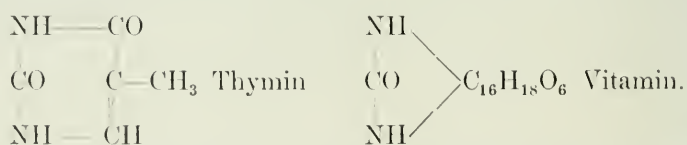


Figur 5. Mikrophotogramm der Beriberi-vitamins Krystalle aus Hefe.

lass das Vitamin sich in der Hefe in gebundenem Zustande befindet. Im ersten Versuch wurden 75 kg getrocknete Hefe mit Alkohol extrahiert, der alkoholische Extrakt im Vakuum eingengt und in kleinen Portionen mit 10% Schwefelsäure fünf Stunden am Rückflusskühler hydrolysiert. Die Fettsäuren wurden abfiltriert, das Filtrat mit demselben Volumen Wasser ver-
lünnt und mit Phosphorwolframsäure gefällt. Die erhaltene Fällung wurde in üblicher Weise zersetzt und die Lösung eingengt. Daraufhin wurde eine konzentrierte wässrige Silbernitratlösung so lange zugesetzt, bis ein Tropfen der klaren Lösung, mit einer wässrigen Barytlösung versetzt, nicht mehr eine weisse, sondern eine braune Fällung von Silberoxyd aufweist. Die von einem sehr voluminösen Niederschlage von Purinbasen befreite Lösung wird abfiltriert und zum Filtrat so lange eine gesättigte Barytlösung zugesetzt,

bis die Flüssigkeit mit einer ammoniakalischen Silbernitratlösung noch einen weissen Niederschlag liefert.

Der so erhaltene Silberniederschlag wurde mit Schwefelwasserstoff zersetzt und das Filtrat nach Entfernung der letzten Spuren Baryts im Vakuum zum kleinen Volumen eingedampft, der Rückstand in eine kleine Schale übergeführt und unter Zusatz von Alkohol im Exsikkator stehen gelassen. Nach einigen Tagen schieden sich Krystalle aus, deren Menge 0,6 g ausmachte. Dieselben wurden aus verdünntem Alkohol umkrystallisiert, wobei 0,45 g in farblosen Nadeln (s. Figur 5) erhalten wurde, die getrocknet bei 233° C schmolzen, also bei derselben Temperatur, wie die Substanz aus Reiskleie. Überhaupt waren die beiden Substanzen in Löslichkeit und anderen Reaktionen vollständig identisch. Vor allen Dingen war auch die Substanz aus Hefe physiologisch wirksam; in Mengen von 2—4 cg kranken Tauben gegeben, vermag sie dieselben in kurzer Zeit zu heilen. Ich halte deshalb die beiden Substanzen für identisch. Die Substanz war durch Quecksilberacetat, aber nicht durch -nitrat und -sulfat fällbar. Sie gab mit einer Nitronlösung versetzt keinen Niederschlag und besass neutrale Reaktion. Mit Kupferoxyd gekocht wurde kein Kupfersalz erhalten, die die Substanz als eine Aminosäure charakterisiert hätte. Was die chemische Natur des Vitamins betrifft, so macht es die Neutralität der wässrigen Lösung, die Unfähigkeit mit Säuren Salze zu geben, sowie die Zugehörigkeit zu der Pyrimidinbasenfraction sehr wahrscheinlich, dass es sich hier um eine neue Pyrimidinbase handelt. Es ist jedenfalls sehr wahrscheinlich, dass die beiden Stickstoffatome im Vitamin ein Ureid bilden, es scheint mir dies die einzig mögliche Erklärung für die indifferente Natur der beiden Stickstoffatome zu sein. Wir wollen deshalb in Analogie zu anderen Pyrimidinbasen die Vitaminformel folgendermassen zergliedern:



Das Filtrat der Silberfällung erwies sich als vollständig inaktiv. Meine Meinung, dass das Vitamin eine Substanz von Pyrimidincharakter ist, wurde durch spätere Versuche bestärkt, die weiter unten ihre Besprechung finden werden. Diese Erkenntnis wird überhaupt für unsere weitere Betrachtung der Nahrungsbestandteile, die für den Lebensvorgang von Bedeutung sind, von grossem Nutzen sein.

Eine andere Hefemenge wurde nach einer abweichenden Vorschrift verarbeitet. Der alkoholische Extrakt der Hefe wurde nämlich nach dem Einengen im Vakuum einfach mit Wasser extrahiert. Dabei ging das Vitamin in die wässrige Lösung über. Dies zeigt, dass man unter die alkohollösliche Substanzen nicht etwa nur Lipide rechnen muss. Im Laufe

dieser Hefeuntersuchung hat sich gezeigt, dass in den Alkohol Stoffe übergehen, die mit Lipoiden nichts zu tun haben, und hauptsächlich zur Purin- und Pyrimidingruppe gehören. Diese Beobachtung ist übrigens auch von Lipoidforschern schon gemacht worden und wird weiter unten ihre Besprechung finden. Wurde nämlich die hier erhaltene wässrige Lösung mit Phosphorwolframsäure gefällt und überhaupt wie oben verfahren, so wurde aus der Silberfraktion nicht Vitamin, sondern eine Substanz isoliert, die mit Uracil identifiziert werden konnte. Da das ganze Vitamin sich im Filtrat befand, so muss geschlossen werden, dass das Vitamin sich in der Hefe im gebundenen Zustand befindet. Dies erklärt auch manche Literaturangaben, die behaupten, dass das Silberverfahren nicht quantitativ die Isolierung des Vitamins gestattet. In solchen Fällen ist es wahrscheinlich, dass das Vitamin teilweise im freien, teilweise im gebundenen Zustande sich befand. Daraufhin wurde das Silbernitratfiltrat mit Schwefelsäure hydrolysiert und das ganze Trennungsverfahren nochmals angewandt. Auch hier wurde ein Gemisch von Pyrimidinbasen vorgefunden, so dass nach einem umständlichen Trennungsverfahren (mit Quecksilbersalzen) nur eine Spur der aktiven Substanz isoliert werden konnte. Aus dieser Fraktion konnte übrigens auch Thymin in reinem Zustand erhalten werden.

Das Problem der Isolierung der aktiven Substanz aus Hefe wurde auch von anderer Seite in Angriff genommen. Edie, Evans, Moore, Simpson und Webster (35) hydrolysierten die Hefe nicht, sondern verarbeiteten den alkoholischen Extrakt nach dem Abdampfen dieses Lösungsmittels direkt mit Silbernitrat und Baryt. Sie erhielten dabei einen hygroskopischen Sirup, der für Tauben sich als sehr wirksam erwies. Dieses Gemisch besserte den Zustand der kranken Tauben in sehr geringen Dosen (3—6 mg). Durch weitere Reinigung wurde eine krystallinische Substanz erhalten, deren geringe Ausbeute eine weitere Reinigung nicht gestattete. Sie analysierten dieselbe, da sie aber noch Asche enthielt, so kommt wohl die Analyse nicht ernsthaft in Betracht. Angaben über die physiologische Wirkung der isolierten Substanz fehlen in der Arbeit. Im übrigen konnten diese Autoren meine Angaben über die verschiedenen von mir angewandten Fällungs- und Isolierungsmittel vollständig bestätigen.

Isolierung des Vitamins aus anderem Rohmaterial.

Es gelang mir auch aus anderen Quellen das Vitamin zu isolieren und wenigstens durch das physiologische Verhalten gegenüber den Polyneuritis-Tauben zu charakterisieren. In den beiden hier weiter unten mitgeteilten Fällen wurde der alkoholische Extrakt oder das Rohlipoid zuerst hydrolysiert. Die angewandte Methode war übrigens identisch mit der zur Untersuchung der Hefe benutzten.

Die Darstellung des Vitamins aus Milch wurde nahezu an 1 kg eines Trockenmilchpräparates ausgeführt, das unter dem Namen „Trumilk“ im Handel bekannt ist.

Nachdem die Wirksamkeit des alkoholischen Extraktes und des Phosphorwolframsäure-Niederschlags festgestellt war, wurde nach der üblichen Methode fraktioniert. Schliesslich wurde eine geringe Menge einer in Nadeln krystallisierenden Substanz enthalten, die bei 230° schmolz und die bekannten Vitamineigenschaften zeigte.

Die Isolierung des Vitamins aus Milch, unserem wichtigsten Nahrungsmittel, werden wir in einem anderen Kapitel, nämlich bei der Barlow'schen Krankheit diskutieren.

Suzuki, Shimamura und Odaka (l. c. 31) fanden allerdings, dass der alkoholische Auszug aus Milch, wie auch die frische Milch, Tauben bei Reisdiet nicht viel länger, als bei Reis allein am Leben erhalten kann, während es bei Mäusen einen sehr günstigen Erfolg gab.

Dass die Milch wirklich Vitamin enthält, ersieht man aus einer sehr interessanten Arbeit von Andrews (36), der gefunden hat, dass die Säuglinge auf den Philippinen meistens an infantiler Beriberi sterben, da sie durch beriberikranke Mütter gestillt werden. Diese Milch unterscheidet sich in ihrer chemischen Zusammensetzung kaum von normaler, sie ist P- und Ca-reicher, dagegen fettärmer. Beim Wechseln der Milch verschwinden alle Symptome bei den schwer kranken Kindern (Erbrechen, Cyanose, Dyspnoe, Tachycardie) binnen wenigen Tagen. (Hirota (37), zit. nach Bältz und Miura, Handb. d. Tropenkrank. B. II). Junge Hunde gehen, mit dieser Milch ernährt, zugrunde.

Auch aus Ochsenhirn (l. c. 32) gelang es mir eine Spur von Vitamin zu isolieren. Die Substanz war leider nicht ganz rein. Die geringe Krystallmenge liess sich nur schwer von der Mutterlauge trennen. Der Schmelzpunkt wurde daher etwas niedriger gefunden (230°). Immerhin besitzt dieser Befund für unsere weiteren Ausführungen einige Bedeutung, auf die wir noch später zurückkommen werden.

Nachweis des Vitamins in manchen Futter- und Genussmitteln.

Das Vitamin ist in unserer Nahrung fast universell verbreitet. In den früheren Abschnitten haben wir schon eine ganze Reihe von Nahrungsmitteln aufgeführt, in denen das Vitamin enthalten ist. Die Anwesenheit dieser Substanz in den weiter unten behandelten Nahrungsmitteln ist allerdings nur durch Tierexperimente am Geflügel festgestellt worden. Da aber das Vitamin eine so spezifische Wir-

lung entfaltet, so muss dies als ein genügender Beweis seiner Anwesenheit betrachtet werden.

So fand Schaumann (l. c. 19), dass das Testikulin des Handels sehr wirksam ist. Thésé fand (38), dass das Handelslecithin wirksam ist, wie wir aber gesehen haben, beruht dies nur auf Verunreinigungen, die bei der Extraktion mit Alkohol mit hinübergehen.

Auch in verschiedenen Zerealien ist das Vitamin aufgefunden worden, so im Hafer, in Weizen und Gerste, in verschiedenen Bohnenarten und auch im Mais.

Von Maclean (39) ist die Substanz im Waschwasser der Lecithinmutterlaugen (aus Fleisch) aufgefunden worden. Suzuki, Shimamura und Odake (l. c. 34) haben gezeigt, dass Hirse und Gemüse die Substanz enthält. Interessant ist ihre Beobachtung, dass entkleiete Gerste, die noch dazu mit Wasser gewaschen wurde, immer noch genug Vitamin enthält, um Tauben am Leben zu erhalten. Es scheint, dass in der Gerste das Vitamin nicht in der Weise an der Oberfläche des Kornes lokalisiert ist, wie beim Reis. Auch im gewöhnlichen Weizenbrot fanden dieselben die aktive Substanz. Die relative Menge von Vitamin, die in verschiedenen Nahrungsmitteln sich vorfindet, wurde auf folgende Weise festgestellt. Die Tauben wurden mit geschältem Reis gefüttert, dazu wurden variierende Mengen von dem zu untersuchenden Futtermittel zugesetzt. Die obengenannten Autoren fanden auf diese Weise, dass die Weizenkleie etwa 10 mal ärmer an Vitamin ist als die Reiskleie; Gerstenkleie etwa 5 mal, Hafer 10 mal, trockener Kohl 10 mal, Adzukibohnen 10 mal ärmer, auch im Gerstenmalz, in Raphanusblättern wurde ihre Gegenwart nachgewiesen.

Eykman (l. c. 21) wies die Gegenwart des Vitamins im Eidotter nach; dagegen haben die japanischen Autoren kein Vitamin darin nachweisen können. Überhaupt stossen wir in der Beriberi-Literatur immerfort auf solche Gegenstände. Das kommt daher, dass das Vitamin sich überhaupt in sehr geringen Mengen findet, und daher kommt es sehr darauf an, wieviel von dem Ausgangsmaterial zum Experiment verwandt worden ist.

Cooper (40) untersuchte auch eine ganze Reihe von Nahrungsmitteln und kam zu folgenden Ergebnissen. Er untersuchte zwei Faktoren, erstens wieviel von dem betreffenden Nahrungsmittel zugesetzt werden muss, um Polyneuritis zu verhüten, zweitens wieviel, um den Gewichtsabsturz zu vermeiden. Die erhaltenen Resultate fasste er in einer Tabelle zusammen, die hier beigegeben sein mag.

Nahrungsmittel	Täglicher Zusatz zur Verhütung der Polyneuritis		Täglicher Zusatz zur Verhütung des Gewichtsverlustes	
	Frisch	Getrocknet	Frisch	Getrocknet
Ochsenfleisch	20 g	5 g	20 g	5 g
Ochsenherz	5 „	2 „	5 „	2 „
Schafhirn	12 „	2,5 „	3—6 „	0,6—1,2 g
Fisch	> 10 „	> 2 „	> 10 „	> 2 „
Eigelb	3 „	1,5 „	10 „	5 „
Presshefe	2,5 „	0,5 „	2,5 „	0,5 „
Linzen	15 „	3 „	30 „	6 „
Gerste ungeschält	3,75 „	3,25 „	7,5 „	6,5 „
„ geschält	5 „	4,5 „	10 „	9 „

Die Quanten sind berechnet für Tauben von 350 g Gewicht, wenn sie $\frac{1}{20}$ ihres Gewichts von poliertem Reis als Nahrung erhalten. Die Versuche zeigen, dass die Nahrungsmittel in bezug auf Gewichtserhaltung und Verhütung von Polyneuritis ungleiche Resultate liefern. Die besten Resultate wurden mit Hefe und Eigelb erhalten.

In welcher Form ist das Vitamin in der Natur vorhanden?

Obwohl wir die Frage schon zum grossen Teil diskutiert haben, müssen wir noch einige Punkte näher betrachten. Mannigfache Untersuchungen haben gezeigt, dass das Vitamin durch Äther und Petroläther nicht extrahierbar ist. Andererseits ist gezeigt worden, dass Wasser, säurehaltiges Wasser, Alkohol und säurehaltiger Alkohol vorzügliche Extraktionsmittel sind. Doch kommt es vor, dass auch diese Lösungsmittel sehr oft nur einen geringen Teil ausziehen, wie es z. B. bei Hefe der Fall ist; Alkohol extrahiert aber eine ganze Anzahl von anderen Substanzen, und man darf auch nicht vergessen, dass Stoffe, die in reinem Zustande in diesem Lösungsmittel vollständig unlöslich sind, darin in Gemengen sich spielend leicht lösen können. Diese Erfahrung ist besonders häufig bei Eiweissderivaten und bei Polypeptiden gemacht worden. Deshalb müssen wir in dem Alkoholextrakt auch ein grosses Gemenge erwarten, was auch tatsächlich zutrifft. Haben doch ausser mir noch Lipoidforscher wie Winterstein und Smolenski (41) und Maclean (l. c. 39) gezeigt, wie unglaublich schwer Phosphatide von manchen stickstoffhaltigen Substanzen (wie ich gezeigt habe, von Nukleinsäurederivaten) zu trennen sind. Auch die unter den Ärzten jetzt so beliebte Darreichung von Phosphorpräparaten beruht wohl lediglich auf den in ihnen eingeschlossenen Verunreinigungen. Andererseits wurde von Fingerling (42) und Abderhalden (43) gezeigt, dass der tierische Organismus wohl imstande ist, seinen Bedarf an organischen Phosphorverbindungen aus anorganischen Phosphaten zu decken. Doch das konstante Vorkommen des Vitamins zusammen mit der Li-

oidfraktion im Alkoholextrakt legte die Vermutung nahe, dass es sich hier um ein Phosphatid handeln könnte. Erst meine Untersuchungen haben klar bewiesen, dass Fraktionen ohne jede Spur von Phosphor heilend wirken können. Ich konnte zeigen, dass es sich um einen stickstoffhaltigen Körper handelt, der aller Wahrscheinlichkeit nach in die Nukleinsäurereihe hineingehört. Höchstwahrscheinlich handelt es sich hier um einen neuen Typus von Pyrimidinbasen oder jedenfalls einer Substanz, die mit dieser Gruppe in engem Zusammenhang steht. Alle meine Bemühungen waren darauf gerichtet, zu untersuchen, ob die Substanz nur in gebundenem Zustand, so wie sie wahrscheinlich in der Natur vorkommt, oder auch im freien Zustande wirksam ist. Meine Hydrolyseversuche wurden angeführt, um diesen Punkt zu entscheiden. Sie haben klar gezeigt, dass nach Spaltung mit einer Säurekonzentration, der keine von den bisher in der Natur beobachteten komplizierten Verbindungen widerstehen konnte, die physiologische Wirkung vollständig erhalten bleibt. Dieser Befund muss besonders betont werden, da er zu der Behauptung von Suzuki, Shimamura und Odake im grossen Gegensatz steht.

Trotzdem ist es sehr wichtig zu erforschen, ob das Vitamin frei oder in einem grösseren Komplex gebunden vorkommt. Die neuesten Ergebnisse von mir und den obengenannten japanischen Autoren berechtigen zu dem Schlusse, dass der letzte Fall zutrifft. Ich glaube durch meine Untersuchungen hergestellt zu haben, dass die Grundsubstanz, das Vitamin, überall dieselbe ist. Habe ich doch gezeigt, dass aus vier verschiedenen Ausgangsmaterialien, wie aus Reiskleie, Hefe, Milch und Gehirn anscheinend dieselbe Substanz isoliert werden konnte. Diese Resultate sind ganz eindeutig. Doch ist es leicht möglich, dass der Komplex, der als solcher wegen des in ihm enthaltenen Vitamins auch heilend wirkt, noch ausserdem andere Bestandteile enthält. Leider stehen uns die Substanzen in so geringen Mengen zur Verfügung, dass wohl noch eine lange Zeit verstreichen wird, ehe alle diese Punkte klargestellt werden. Immerhin konnte ich zeigen, dass das Vitamin in der Hefe in gebundenem Zustande vorkommt, die Liverpoolsche Schule (mit Moore) konnte dasselbe sehr wahrscheinlich machen, indem sie in der heilenden Fraktion eine Substanz nachweisen konnte, die durch Spaltung Trimethylamin lieferte. Die japanischen Forscher (Suzuki) machten es für die Reissubstanz wahrscheinlich, indem sie durch Spaltung der Rohsubstanz zwei verschiedene stickstoffhaltige Substanzen und ausserdem Cholin und Glukose isoliert haben.

Was die Stabilität gegen höhere Temperatur betrifft, so ist dieselbe nicht so gering, wie man nach den Ergebnissen von Gryns erwarten sollte. Konnte ich doch zeigen, dass sogar nach 20stündigem Erhitzen mit Schwefelsäure die heilende Wirkung teilweise noch erhalten bleibt. Immerhin haben alle Untersucher die unangenehme Erfahrung gemacht, dass während der

Fraktionierung, besonders am Ende derselben der grösste Teil der Substanz verloren geht¹⁾. Es wäre wichtig, den Grund dieses Verhaltens zu finden. Es wäre nicht unmöglich, dass er in der Anwendung des Alkalis zur Eliminierung der Phosphorwolframsäure oder des Tannins zu suchen ist. Ich suchte, dies zu vermeiden, indem ich gleich eine Fällung mit Silbernitrat und Baryt vornahm, in welchem Falle nur sehr wenig Alkali angewandt wird. Leider stiess ich hier auf Schwierigkeiten, indem ich Stoffe fand, die bei Anwendung der gewöhnlichen Methode ins Filtrat übergehen (Auffindung von Allantoin in der Reiskleie). Ich glaube, dass eine Methode, die das Vitamin in wenigen Manipulationen ohne Anwendung von starkem Alkali zu isolieren erlaubte, die Ausbeute an diesem Stoff ganz bedeutend steigern würde.

Die Untersuchungen in der Beriberifrage werfen auch ein neues Licht auf die Chemie der Lipoide, indem sie uns Mittel geben, die Reinheit derselben zu prüfen. Wir besitzen dafür zwei Methoden: eine biologische und eine chemische. Die erste beruht darauf, dass unreine Lipoide Geflügel-Polyneuritis zu heilen vermögen. Zeigt sich ein Lipoid mittels der sehr empfindlichen Reaktion als frei von Vitamin, so können wir ruhig schliessen, dass das isolierte Lipoid auch frei von anderen Verunreinigungen ist. Eine zweite Methode beruht darauf, dass die gewöhnlichen Verunreinigungen zu der Klasse der Purin- und Pyrimidinsubstanzen gehören. Als solche können sie mit Silbernitrat allein oder mit Silbernitrat und Baryt nachgewiesen werden. Wird die Lipoidfraktion mit Wasser ausgekocht und können im Waschwasser die obengenannten Substanzen nicht nachgewiesen werden, so ist wohl die Substanz als rein zu betrachten. Wir werden jetzt kaum ein Phosphatid als rein erklären wenn diese Prüfungen nicht vorgenommen worden sind. Es ist zu hoffen, dass dies die Lipoidchemie sehr vereinfachen wird.

Ein Versuch das Wesen der Beriberi-Krankheit zu erklären.

Wie wir aus dieser Zusammenfassung ersehen können, wissen wir über die chemische Natur der fehlenden Substanz mehr, als über das Wesen der Krankheit. Diese ist für uns noch ein vollständiges physiologisches Rätsel geblieben. Wir sind leider hier nur auf Vermutungen angewiesen. Auf die Intoxikations- und Infektions-Theorien wollen wir hier nicht eingehen, da sie meiner Meinung nach durch keine Beweise gestützt sind. Die meisten modernen

¹⁾ Übrigens kennen wir schon eine thermolabile Substanz: nämlich Allantoin. Dieselbe gibt in reinem Zustande nach 1 Min. langem Kochen mit Triketohydrindenhydrat (Abderhalden und Schmidt, Über die Verwendung von Triketohydrindenhydrat. Zeitschr. f. physiol. Ch. 72, 87, 1911) keine Färbung. Die Färbung tritt aber beim längeren Kochen (2 bis 3 Min.) auf und ebenso wenn Allantoin mit Wasser oder besser mit Bleisuperoxyd am Rückflusskühler erhitzt wird. Ob die Färbung auf das Entstehen der Allantoin-säure zurückzuführen ist, konnte ich noch nicht entscheiden.

beriberi-Forscher akzeptieren die partielle Unterernährung als die einzige Ursache der Krankheit. Vergl. u. a. die grundlegende Arbeit von Strong (44) und Crowell, die die Frage ausführlich behandelt. Allerdings kommen von Zeit zu Zeit Meinungen zum Vorschein, wie die vor kurzer Zeit erschienene von Shibayama (45), der in Beriberi ausser dem Diätfaktor noch eine Infektion zu erblicken sucht.

Fassen wir das zusammen, was wir über die Ätiologie dieser Krankheit bisher wissen, so finden wir, dass Beriberi nur dann ausbricht, wenn eine vitaminarme oder vitaminfreie Nahrung durch lange Zeitperioden hindurch genossen wird. Als solche einseitige Nahrung können wir auf Grund unserer Erfahrung polierten Reis und Weissbrot bezeichnen. Dass darin nur das Vitamin fehlt, ersieht man daraus, dass, wenn zum Reis nur ein wenig Gärkhefe, Hefe oder Bohnen zugesetzt wird, die Patienten in derselben Nahrung alles finden, was sie zum Lebensprozess benötigen. Nun müssen wir uns die Frage stellen, welche physiologische Bedeutung das Vitamin besitzt. Dass diese Substanz eine sehr grosse Rolle im Organismus spielt, ergibt sich aus der Schwere der Symptome, die durch ihr Fehlen verursacht werden. Man kann mit voller Sicherheit behaupten, dass das Leben mancher Tiere, und vor allem des Menschen, auf die Dauer unmöglich ist, wenn das Vitamin in der Nahrung vollständig fehlt. Wie wir allerdings aus der Liste der Nahrungsstoffe ersehen, in denen die Gegenwart des Vitamins nachgewiesen worden ist, sind wir bei normaler Ernährung wohl kaum der Beriberigefahr ausgesetzt. Das gilt jedoch nur für unsere kleinen und für Erwachsene. Da die Ernährung der Säuglinge sehr einseitig ist, so kommen sie viel mehr in die Gefahr unter dem Mangel an Vitamin zu leiden; wir werden dies noch ausführlich bei der Barlowschen Krankheit besprechen. Doch nicht jede einseitige Ernährung bringt die Beriberigefahr mit sich. Wir sehen Bauern in Russland und in Irland sich fast ausschliesslich mit Kartoffeln ernähren, ohne dass irgendwelche Schäden daraus entstehen. Die europäischen Völker haben nämlich mit ihrem Instinkt herausgefunden, dass die Kartoffeln alles bieten, was zur Erhaltung des Lebens nötig sei. Seit der Einführung der Kartoffel in Europa, sind die grossen Skorbutepidemien, die im Mittelalter hauptsächlich in den Städten viele Opfer forderten, vollständig erloschen. Eine Kartoffelzulage zur Reismahlung könnte meiner Meinung nach segensreich in den Beriberizonen wirken.

Von den Beriberi-Symptomen sind es die nervösen Störungen, die uns am meisten auffallen. Der Gewichtssturz scheint nach neueren Erfahrungen ein konstantes Vorkommnis zu sein. Auch von Versuchstieren ist dasselbe behauptet worden. Ich konnte jedoch für Tauben dies nicht bestätigen. Wurde nämlich der mit Reis gefüllte Kropf entleert, so konnte regelmässig ein Gewichtssturz notiert werden. Ob dagegen eine grössere

Reiszufuhr den Ausbruch der Krankheit beschleunigt oder verlangsamt, darüber sind die Autoren nicht einig. Während Cooper behauptet, dass das erste der Fall ist, sind Chamberlain, Bloomleigh und Kilbourne (46) der Meinung, dass die Krankheit durch Unterernährung schneller hervorgerufen wird und ich schliesse mich dieser Meinung an. Sie behaupten sogar bei hungernden Tauben typische Polyneuritis gesehen zu haben. Ich kann dies zum Teil bestätigen. Werden nämlich Tauben nicht künstlich ernährt, sondern einfach der Reis in den Käfig gestreut, so fressen die Tiere den Reis am Anfang sehr gierig, nach etwa einer Woche verlieren sie jedoch gänzlich den Appetit und hören mit dem Fressen auf. Kommt so eine Taube zur Sektion, so wird im Kropf nie ein Reiskorn aufgefunden. Die meisten von diesen Tauben sterben an allgemeiner Schwäche, doch in etwa 40% aller Fälle sieht man eine typische Polyneuritis sich entwickeln.

Was die nervösen Störungen anbetrifft, so war ich bemüht dafür eine plausible Erklärung zu finden, und suchte meine Vermutung auch experimentell zu stützen. Ich stelle mir den Vorgang folgendermassen vor.

Wird das Versuchstier mit poliertem Reis ernährt, so sucht es das ihm unentbehrliche Vitamin auf irgendeine Weise zu beschaffen. Nun besitzt es in der Regel einen Vorrat, der von früherer normaler Diät stammt, dieser wird nun mobilisiert und für die Stoffwechselvorgänge verwendet. Zuerst kommen wohl Organe in Betracht, wie z. B. die Muskeln, deren physiologische Rolle nicht so bedeutend ist. Ist dieser Vorrat erschöpft, so werden andere Organe dieser Substanz beraubt. Steht dem Tier nichts mehr zur Verfügung, so werden schliesslich das Gehirn, in welchem ich das Vitamin nachgewiesen habe, und die Nerven herangezogen. Nun scheint es gerade, dass in diesen Organen das Vitamin eine grosse Rolle spielt. Es treten dann die charakteristischen Symptome auf, die das bekannte Bild der Beri-beri und Polyneuritis darstellen. In der Tat finden wir in den Nerven und im Herzen eine fettige Degeneration, die nie ausbleibt. Nun wissen wir gerade, dass das Vitamin in den Organen besonders lokalisiert ist, die uns als lipoidreich bekannt sind. Das Vitamin ist wenigstens biologisch, wenn nicht chemisch mit den Lipoiden assoziiert.

Dass die Tiere nur nach einer gewissen Zeit der Krankheit verfallen, die in einzelnen Fällen sehr variiert, findet vielleicht seine Erklärung in dem Vorrat an Vitamin, das in den Organen aufgestapelt ist.

Ich suchte nun experimentell festzustellen (Casimir Funk [47]), ob sich in den Organen der kranken Tiere chemische Differenzen gegenüber den normalen feststellen lassen. Zu diesem Zwecke wurden Taubenhirne analysiert, und zwar auf Stickstoff und Phosphor, Elemente, die wohl zusammen den Gehalt an Lipoiden gut genug anzeigen. Ich habe in dieser Richtung normale Tiere, sowie eine Anzahl Polyneuritis-Tauben untersucht. Zum Vergleich habe ich auch Fälle untersucht, in welchen Tiere

mit verschiedenen Mitteln geheilt worden sind. Als Kontrolle wurden Tauben benutzt, die mit einer Maismenge gehalten wurden, die stark unter dem normalen Bedarf lag. Als diese soviel an Gewicht verloren hatten, wie die Polyneuritis-Tauben wurden sie getötet und das Gehirn analysiert. Die Resultate mögen in einer Tabelle wiedergegeben werden.

	Anzahl der Tiere	An- fangs- gewicht	End- gewicht	Hirn feucht	Hirn trocken	N%	P%	N in mg	P in mg
Normal	8	—	320	1,8	0,3848	9,77	1,84	37,6	7
Polyneuritis	12	300	232	1,75	0,3602	9,31	1,53	33,7	5,5
Geheilte	6	314	259	1,82	0,3914	9,37	1,57	36,7	6,1
Unterernährung . .	3	352	269	1,98	0,4330	9,62	1,85	41,7	8

Wie aus der Tabelle ersichtlich ist, gegenüber den normalen Tieren, ist der P- und N-Gehalt des Gehirnes bei Polyneuritis bedeutend niedriger, was einen Abbau der Phosphatide sehr wahrscheinlich macht. Bei den geheilten Tieren, die 24 Stunden nach der Kur getötet worden sind, scheint der normale Zustand noch nicht eingetreten zu sein. Die unterernährten Tiere zeigen keine merkliche Verminderung dieser beiden Elemente. Ist es doch eine in der Physiologie lange bekannte Tatsache, dass im Hunger das Gehirn das letzte Organ ist, das Verluste erleidet. Wieland (48) scheint allerdings anderer Meinung zu sein. Auf die Arbeit Palladinos (49) sich stützend, der bei hungrigen Hunden (bloss ein Experiment und eine Kontrolle) im Gehirn, grosse allgemeine Verarmung an organischen Bestandteilen feststellte, glaubte er, dass es sich in meinem Falle um Hungerfolgen handelt. Im übrigen finde ich meine Ansicht in seinen Experimenten bestätigt, da er bei Mäusen, die mit poliertem Reis ernährt worden sind, eine sehr deutliche Abnahme des organisch gebundenen Phosphors feststellen konnte, während der gesamte Phosphor eher anstieg. Dies ist im Einklang mit den klinischen Beobachtungen an Beriberi-Patienten, bei welchen eine Vermehrung der Phosphorausscheidung im Harn oft konstatiert worden ist. Wieland fand bei normalen Mäusen im Mittel 0,74% Gesamtphosphor des Trockengewichtes, während bei ernährungskranken 0,89% gefunden wurde; für den organisch gebundenen Phosphor waren die Zahlen: 0,19% für gesunde, 0,17% für kranke Tiere.

Es ist fraglich, ob meine Auffassung von der Ursache des Auftretens nervöser Symptome durch meine Arbeit streng bewiesen ist. Trotzdem glaube ich, dass die von mir gegebene Erklärung zur Zeit die plausibelste ist. Das ganze Bild der Krankheit bleibt nichtdestoweniger ein Rätsel. Es ist fraglos, dass der Mangel an Vitamin den ganzen Stoffwechsel beeinträchtigt. Es muss daher dem Vitamin noch irgendeine wichtige physiologische Funktion zugeschrieben werden. Die kleine Substanzmenge, die hier ins Spiel tritt, kann wohl kaum vom Standpunkt des Energie- oder Kalorienwertes betrachtet

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Eastman Park,
werden. Ich habe schon früher (l. c. 22) die Ansicht ausgesprochen, dass das Vitamin eine Ausgangssubstanz für die Bereitung gewisser, im Stoffwechsel nötiger Substanzen sein muss. Als solche kommen Hormone, Fermente und die Sekrete der Drüsen ohne Ausführungsgang in Betracht. Eine Stütze für meine Anschauung bringt die Arbeit von Watson (50). Watson fand bei jungen Mäusen und Ratten, bei ausschliesslicher Mehlnahrung, Degeneration der Schilddrüse, während die Kontrollen bei Milehnahrung gesund blieben. Vedder (51) gelang es aber nicht Tiere durch Zufuhr der Schilddrüsensubstanz vor Polyneuritis zu schützen. Wir werden im Laufe unserer Ausführungen sehen, dass zu dieser Gruppe von Substanzen noch manche Stoffe zu zählen sind, deren Untersuchung leider noch kaum angefangen ist. Schaumann (l. c. 33) sprach sich für eine ähnliche Auffassung aus, da er aber seine Phosphormangelhypothese noch nicht ganz verlassen will, so nimmt er an, dass das Vitamin bei der Synthese organischer Phosphorverbindungen eine Rolle spielt. Für diese Auffassung fehlen jedoch jegliche Beweise.

Im Verlauf meiner Untersuchungen stiess ich auf Tatsachen, die, wenn sie auch keine Ansprüche auf die Lösung des Problems erheben, doch meiner Meinung nach manches dazu beitragen können. Obwohl die Untersuchungen noch nicht abgeschlossen sind, möchte ich sie kurz skizzieren. Bei der Fraktionierung der Reiskleie (l. c. 32) konnte ich vor kurzem Allantoin isolieren. Diese in so mannigfachen Beziehungen der Harnsäure nahestehende Substanz zeigt grosse Ähnlichkeit mit dem Vitamin. Ich habe dieselbe auch Polyneuritis-Tauben verabreicht und habe sehr interessante Resultate erhalten. Wird das synthetische Präparat in kleinen Mengen gegeben, so zeigt sich nach einigen Stunden eine sehr deutliche Besserung. Während in der Regel Tauben in diesem Stadium nach 6—12 Stunden sterben, konnte ich diese Tiere durch tägliche Gaben dieser Substanz einige Tage am Leben erhalten. Ich habe eine ganze Anzahl solcher Versuche ausgeführt und konnte meine Beobachtung vollauf bestätigen. Dies ist um so merkwürdiger, als allgemein angenommen wird, dass das Allantoin sich leicht aus Harnsäure bilden kann.

Harnsäure ist bekannterweise ein Endprodukt im Stoffwechsel der Vögel und wird als solche ausgeschieden. Ich kam daher zur Ansicht, dass Allantoin von Tauben nicht aus Harnsäure bereitet werden kann, da sonst diese Substanz kaum eine Wirkung entfalten könnte. Ich gab daher den kranken Tauben Harnsäure und sah, dass diese ohne Wirkung war. Bei Durchsicht der einschlägigen Literatur fand ich übrigens meine Ansicht vollständig bestätigt. Battelli und Stern (52) geben in diesen Ergebnissen der Physiologie an, dass die einzigen Tiere, die in ihren Organen kein urikolytisches Ferment (Urikooxydase) aufweisen, die Vögel und der Mensch seien. Es ist auffallend, dass dies ebenfalls die einzigen Tiere sind, die für Gicht veranlagt sind. Es ist auch sehr auffallend, dass sie zugleich die einzigen Tiere sind, die durch Zufuhr von weissem Reis typische Polyneuritis (Beriberi) be-

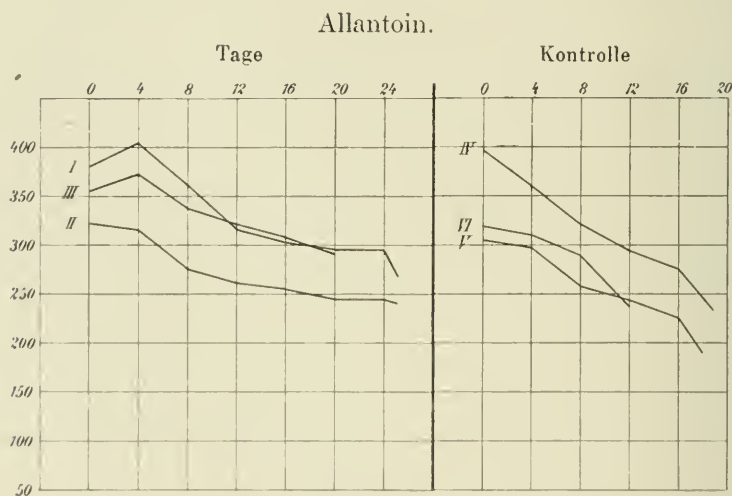
kommen. Es wäre vergeblich, bei einem Allantoin führenden Tiere, das heisst bei solemem, das auf eine Zufuhr von Harnsäure oder Purinen mit einer vermehrten Allantoinausscheidung im Harn reagiert, Polyneuritis erzeugen zu wollen. Werden diese Tiere auf eine spezifisch ungenügende Nahrung gesetzt, so erkranken sie unter Symptomen, die an Skorbut erinnern. Zu solchen Tieren gehören Kaninchen, Meerschweinchen, Hunde, Mäuse, Ratten und wahrscheinlich Affen.

Es muss nun die Frage aufgeworfen werden, woher die Spuren von Allantoin kommen, die von Wiechowski (53) mit einer verfeinerten Methode konstant im Menschenharn gefunden worden sind. Die Tatsache, dass in menschlichen Organen keine Urikooxydase aufgefunden werden konnte, zusammen mit der, dass nach Zufuhr von Harnsäure und Purinderivaten keine Vermehrung der Allantoinausscheidung stattfindet, zeigt, dass der Mensch unfähig ist, Allantoin zu bilden. Die Spuren dieser Substanz, die im Harn aufgefunden worden sind, stammen aus der Nahrung und nicht durch Abbau der Harnsäure, wie Wiechowski behauptet. Der Ursprung des Allantoins war so rätselhaft, dass Abderhalden und Einbeck (54) und dieselben und Schmid (55) versucht haben, ob nicht die Histidinzufuhr bei Hunden eine vergrösserte Allantoinausscheidung im Harn hervorrufe, übrigens mit negativem Resultat. Allantoin wurde schon oft in unserer Nahrung nachgewiesen. So fanden Richardson und Crampton (56) Allantoin in Weizenkeimen, Lippmann (57) in Rübensaft, Smolenski (58) in der Zuckerrübe und Ackroyd (59) in Milch und in anderen Nahrungsmitteln in folgenden Mengen: Milch 0,199 g im Liter, Bohnen 0,078 %, Weissbrot 0,006 g im Kilo, Erbsen 0,0002 %. Dagegen nicht in Eiern, Bananen, Rhabarber. Ackroyd glaubt, dass die Allantoinspuren, die man im Harn findet, den geringen Mengen entsprechen, die in der Nahrung vorhanden sind. Ich schliesse mich hier vollständig der Ansicht Ackroyds an. Die Frage kann natürlich auch experimentell in Angriff genommen werden. Da wir im polierten Reis eine Nahrung haben, der Allantoin offenbar fehlt, so müsste bei dieser Ernährung das Allantoin aus dem Harn verschwinden. Ein Ausbruch der Beriberi ist natürlich nicht bei kurzen Versuchsperioden zu befürchten, da beim Menschen diese Krankheit erst nach 6 bis 7 monatlicher ausschliesslicher Ernährung mit weissem Reis zum Ausbruch kommt. Wir verstehen deshalb, warum verschiedene Tiere verschieden auf Reishaltung oder auf eine andere ungenügende Nahrung reagieren. Die Untersuchungen von Schittenhelm (60), Jones (61) haben klar bewiesen, dass die einzelnen Tierarten in bezug auf ihren Purinstoffwechsel und auf die Verteilung der dazu gehörenden Fermente stark differieren. Mensch und Vögel sind nicht imstande Allantoin zu bilden, Schweine dagegen sind nicht imstande, Guanin anzugreifen, und weisen die sog. Guaninicht auf.

Die Beobachtung, die ich bezüglich der Allantoinwirkung auf kranke Tauben machte, habe ich schon früher bei einer Fraktion aus Zitronensaft

(lime-juice) (l. c. 22) gemacht. Wir werden später beim Skorbut sehen, dass auch dies nur auf die Gegenwart eines Nukleinsäurederivats zurückzuführen ist. Ich habe auch Versuche ausgeführt, bei welchen zum polierten Reis eine tägliche Zulage von Allantoin gemacht wurde. Es wurden 3 Tauben als Kontrolle benutzt, 3 Tauben erhielten dagegen einen täglichen Zusatz von 0,025 g Allantoin. Beide Versuchsreihen wurden mit 30 g Reis täglich künstlich gefüttert. Die Resultate waren wie folgt: die Allantointiere lebten länger und zeigten viel geringeren Gewichtsverlust als die Kontrolltiere. Bei einem ähnlichen Versuch mit Hydantoin wurde eine geringe Lebensverlängerung, dagegen ein viel geringerer Gewichtsverlust erreicht. (Vergleiche dazu die beigelegten Gewichtskurven, Figur 6.) Die Arbeit erscheint demnächst im Journ. of Physiol. (45. 489. 1913).

Nun wurde eine ganze Reihe von Purin-, Pyrimidin- und Nukleinsäurederivaten auf ihre Wirkung gegenüber den Polyneuritistauben geprüft. Die Versuche wurden ausgeführt erstens um zu erfahren, ob diese lebensverlängernde Wirkung auf das Vorhandensein gewisser chemischer Gruppen zurückzuführen ist und zweitens, um zu erforschen, ob diese Wirkung uns nicht irgendwelche Anhaltspunkte über die chemische Struktur des Vitamins liefern könnte. Die Versuche wurden so ausgeführt, dass Tauben, bei welchen durch Reiszufuhr Polyneuritis erzeugt worden war, nach Ausbruch der typischen Symptome, der zu untersuchende Stoff per os eingeführt wurde. Als Dosis wurde gewöhnlich 0,1 g benutzt. Die meistens schwer zu beschaffenden Substanzen habe ich mir nur zum geringen Teil selbst dargestellt, ich verdanke dieselben der grossen Liebesswürdigkeit von Dr. Levene vom Rockefeller-Institute, Prof. Winterstein-Zürich und Prof. Schittenhelm-Königsberg.



Figur 6.

Versuchstiere.	Kontrolltiere.
Tauben: 30 g polierter Reis und 0,025 g Allantoin täglich.	30 g Reis (poliert).

Purinsubstanzen.

Substanz	Formel	Tägliche Dosis g	Zahl der Tiere	Ursprung des Präparats	Resultate
Harnsäure	$ \begin{array}{c} \text{NH}-\text{CO} \\ \quad \\ \text{CO} \quad \text{C}-\text{NH} \\ \quad \quad \diagup \\ \text{NH}-\text{C}-\text{NH} \quad \text{CO} \end{array} $	0,2	2	Kahlbaum	Keine Wirkung
Adenin	$ \begin{array}{c} \text{N}-\text{C} \cdot \text{NH}_2 \\ \quad \\ \text{CH} \quad \text{C}-\text{H} \\ \quad \quad \diagup \\ \text{NH}-\text{C}-\text{N}=\text{CH} \end{array} $	0,1	4	Winterstein	30–50 Stunden
Guanin	$ \begin{array}{c} \text{NH}-\text{CO} \\ \quad \\ \text{NH}_2-\text{C} \quad \text{C}-\text{NH} \\ \quad \quad \diagup \\ \text{N} \quad \text{C}-\text{N}=\text{CH} \end{array} $	0,1 Sulfat	2	Levene	Überleben 24–30 Stunden
Hypoxanthin	$ \begin{array}{c} \text{NH}-\text{CO} \\ \quad \\ \text{CH} \quad \text{C}-\text{NH} \\ \quad \quad \diagup \\ \text{N} \quad \text{C}-\text{N}=\text{CH} \end{array} $	0,1	2	Winterstein	3–5 Tage
Xanthin	$ \begin{array}{c} \text{NH}-\text{CO} \\ \quad \\ \text{CO} \quad \text{C}-\text{NH} \\ \quad \quad \diagup \\ \text{NH}-\text{C}-\text{N}=\text{CH} \end{array} $	0,1	2	Winterstein	2–4 Tage
Paraxanthin	$ \begin{array}{c} \text{CH}_3-\text{N}-\text{CO} \quad \text{CH}_3 \\ \quad \\ \text{CO} \quad \text{C}-\text{N} \\ \quad \quad \diagup \\ \text{NH}-\text{C}-\text{N}=\text{CH}_3 \end{array} $	0,1	2	Winterstein	Sehr deutliche Wirkung und Heilung für 5 Tage, aber nur in einem Falle.
Theophyllin	$ \begin{array}{c} \text{CH}_3-\text{N}-\text{CO} \\ \quad \\ \text{CO} \quad \text{C}-\text{NH} \\ \quad \quad \diagup \\ \text{CH}_3-\text{N}-\text{C}-\text{N}=\text{CH} \end{array} $	0,1	2	Winterstein	Toxische Wirkung

Pyrimidine.

Substanz	Formel	Tägliche Dosis g	Zahl der Tiere	Ursprung des Präparats	Resultate
Uracil	$ \begin{array}{c} \text{NH}-\text{CO} \\ \quad \\ \text{CO} \quad \text{CH} \\ \quad \\ \text{NH}-\text{CH} \end{array} $	0,1	2	Eigenes Präparat	Deutliche Wirkung. 67–100 Stunden am Leben.
Thymin	$ \begin{array}{c} \text{NH}-\text{CO} \\ \quad \\ \text{CO} \quad \text{C}-\text{CH}_3 \\ \quad \\ \text{NH}-\text{CH} \end{array} $	0,05	2	Levene	Keine Wirkung 1–2 Tage

Andere Substanzen, die mit den Purinen im engem Zusammenhang stehen.

Allantoin	$ \begin{array}{c} \text{NH}-\text{CO} \quad \text{CH}_2 \\ \quad \quad \\ \text{CO} \quad \quad \text{CO} \\ \quad \quad \\ \text{NH}-\text{CH}-\text{NH} \end{array} $	0,1	7	Eigenes Präparat	Sehr deutliche Wirkung. 48 bis 79 Stunden am Leben
Alloxantin	$ \begin{array}{c} \text{NH}-\text{CO} \quad \text{CO NH} \\ \quad \quad \\ \text{CO} \quad \text{C(OH)} \quad \text{C(OH)} \\ \quad \quad \\ \text{NH}-\text{CO} \quad \text{CO}-\text{NH} \end{array} \begin{array}{c} \diagup \quad \diagdown \\ \text{CO} \end{array} $	0,1	2	Winterstein	3 Tage am Leben
Hydantoin	$ \begin{array}{c} \text{NH}-\text{CO} \\ \\ \text{CO} \\ \\ \text{NH}-\text{CH}_2 \end{array} $	0,2	5	Eigenes Präparat	Sehr deutliche Wirkung. 2 bis 9 Tage am Leben

Nukleinsäuren und Nukleoside.

Hefenukleinsäure	$\text{C}_{33} \text{H}_{49} \text{N}_{15} \text{O}_{29} \text{P}_4$	1	1	Böhringer	4 Tage am Leben
Thymusnukleinsäure	$\text{C}_{43} \text{H}_{51} \text{O}_{30} \text{N}_{15} \text{P}_4$	0,2	2	Schittenhelm	10—15 Tage am Leben
Guanosin	$\text{C}_{10} \text{H}_{13} \text{O}_5 \text{N}_5$	0,1	3	Levene	1 bis 4 Tage am Leben
Adenosin	$\text{C}_{10} \text{H}_{13} \text{O}_4 \text{N}_5$	0,05	2	Levene	4 Tage am Leben
Cytidin	$\text{C}_9 \text{H}_{13} \text{O}_5 \text{N}_3$	0,05	2	Levene	2—6 Tage am Leben
Uridin	$\text{C}_9 \text{H}_{12} \text{O}_6 \text{N}_2$	0,05	2	Levene	3—10 Tage am Leben

Bei der Betrachtung der erhaltenen Resultate fallen uns hauptsächlich folgende Punkte auf. Die Purinsubstanzen haben wenig oder keine Wirkung. Wirksam sind vor allem die Pyrimidine, sowie Komplexe, die dieselben enthalten. Sehr wirksam sind auch Allantoin und Hydantoin. Die Tiere mit den wirksamen Substanzen behandelt, zeigen Perioden, die fast an echte Heilung erinnern. Der einzige Unterschied ist, dass die Besserung höchstens einige Stunden dauert, die Tiere bleiben dann gewöhnlich einige Tage am Leben, nur vorübergehend eine Besserung (meistens in einigen Stunden nach der erneuten Gabe) zeigend. Mit Paraxanthin erhielt ich Heilungen, die 5 Tage andauerten. Ich möchte die Resultate dahin interpretieren, dass diese Substanzen, mit einer strukturellen Ähnlichkeit zum Vitamin, dasselbe in einigen Funktionen ersetzen können. Meiner Meinung nach ist dies ein neuer Beweis für die Zugehörigkeit des Vitamins zu der Nukleinbasenreihe.

Es muss noch hervorgehoben werden, dass eine ähnliche Wirkung, wie die der Pyrimidinderivate, auch bei Anwendung gewisser Alkaloide gefunden wurde. So gibt Cooper (l. c. 40) an, dass das Strychnin eine verlängernde Wirkung auf das Leben der Polyneuritistauben aufweist. Diese Wirkung aber, die höchstwahrscheinlich auch anderen Alkaloiden zukommt, ist meiner Meinung nach auf eine ganz andere Ursache zurückzuführen. In diesem Falle handelt es sich wohl nur um eine Stimulation des abgeschwächten Nervensystems.

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Skorbutgruppe.

(Skorbut, experimenteller Skorbut bei Tieren, Schiffberiberi und Barlowsche Krankheit.)

Diese ganze Reihe von Krankheiten bildet unzweifelhaft eine Gruppe, da sie auf analoge Ursachen zurückzuführen sind. Von diesen ist Skorbut die best bearbeitete. Unser ganzes Wissen über experimentellen Skorbut verdanken wir fast ausschliesslich Axel Horst und seinem Mitarbeiter Fröhlich.

Es ist wohl jetzt schon allgemein angenommen, dass Skorbut mit der Nahrung kausal zusammenhängt. Die Krankheit bricht aus, wenn eine Nahrung ausschliesslich aus Brot, Stärke und Konserven besteht. Als Heilmittel, die schon vor langer Zeit von Seeleuten empirisch gefunden worden sind, gelten noch immer gute diätetische Bedingungen, Zitronensaft (lime-juice), frisches Obst und Gemüse. Werden die Patienten in dieser Weise behandelt, so werden sie in kurzer Zeit vollständig gesund¹⁾.

Hier, wie bei Beriberi war die Entdeckung der experimentellen Form bei Tieren von Holst und Fröhlich (62) von ganz ausschlaggebender Bedeu-

¹⁾ Historische Angaben über die Antiskorbutica, zu denen auch senfführende Pflanzen (ochlearia) gerechnet werden, finden wir bei Schelenz (Scharbock), Arch. f. Schiffs- und Tropenhyg. 16. 113. 1912).

tung. In ihrer klassischen Arbeit zeigten sie, dass manche Tiere (Meerschweinchen, Kaninchen, Schweine und Hunde), die mit einer Nahrung gefüttert werden, die bei Menschen Skorbut hervorruft, oder auch mit einer Nahrung, die im Autoklaven hohen Temperaturen ausgesetzt worden war, einer Krankheit anheimfallen, die mit dem menschlichen Skorbut, wenn nicht ganz identisch, so doch sicher vollständig analog zu sein scheint. Sie haben gefunden, dass Meerschweinchen, die mit Roggen- oder Weizenbrot und Wasser auch mit Hafer-, Roggen-, Weizen-, Gersten- und Reismehl oder mit Hafer-, Gerste- und Reisgruppen gefüttert werden, nach einigen Wochen eingehen. In Fällen, wo der Tod erst nach drei Wochen eintrat, wurden konstant folgende Veränderungen aufgefunden. Die Mahlzähne wurden lose, mit Blutungen im Zahnfleisch. Manchmal wurde nur eine bläuliche Hyperämie beobachtet und in wenigen Fällen Ulzerationen; Blutungen wurden ausserdem konstant in den Weichteilen der Kniegegend und unter dem Periost der vorderen Enden der Rippen gefunden. Öfters wird die Verbindung zwischen Rippen und Knorpel gelockert, oft auch Ablösungen der Epiphysen der Röhrenknochen, besonders der Tibiae gefunden. Ferner wurden Knochenbrüchigkeit, Duodenalgeschwüre, Haematurie, Oedeme beobachtet. Die mikroskopischen Befunde sind mit denen bei Morbus Barlow identisch: Ossifikationsstillstand, sowie Atrophie des fertigen Knochengewebes an den Ossifikationszonen; das Knochenmark verliert (an den Diaphysenenden) seinen lymphoiden Charakter, wird zellarm, mitunter homogen („Helles Mark“). Beim Schwein entsteht neben Skorbutläsionen öfters eine erhebliche Polyneuritis mit Lähmungen, also Skorbut mit Beriberisymptomen. Von Holst (63) wurden auch Skorbutfälle beim Menschen beobachtet, die geringe Beriberisymptome zeigten. Für die Skorbutuntersuchungen werden von Holst besonders Meerschweinchen empfohlen, die auf Hafer und Wasser gesetzt werden. Zur Anwendung sollen nur erwachsene Tiere kommen, deren Gewicht 350 g übersteigt. Die Tiere sollen alle paar Tage gewogen werden, sie verlieren nämlich sehr stark an Gewicht (bis zu 40%).

Ähnliche Experimente wurden auch von Schaumann (l. c. 19) ausgeführt. Dieser arbeitete speziell mit Hunden und Affen, welche entweder mit Nahrung von Segelschiffen, die von Schiffberiberi befallen waren, oder mit Nahrung, die mit Natriumkarbonat extrahiert worden war, gefüttert wurden. Er beschreibt Symptome, die er als Neuritis bezeichnet. Nach Holst sind wohl die Symptome total von Geflügel-Polyneuritis verschieden und müssen als Skorbut bezeichnet werden.

In einer neuen Arbeit (64) bestätigen Holst und Fröhlich ihre früheren Angaben und erweitern das Tatsachenmaterial sehr bedeutend. Auf Grund ihrer Versuche kommen sie zu dem Ergebnis, dass wohl kein Grund vorliegt, für den experimentellen Skorbut eine andere Ursache anzunehmen, als für den menschlichen Skorbut. Die Symptome traten nicht ein, wenn

erschweinchen mit Weisskohl, Karotten oder Löwenzahn gefüttert wurden, auch dann nicht, wenn die Tiere unter einem grossen Gewichtsverlust (30—40%) verendeten. Andererseits liess sich nachweisen, dass die Tiere nach Getreidekost schon zu einer Zeit skorbutische Veränderungen zeigten, wo noch keine Abmagerung stattgefunden hatte, es ist deshalb ausgeschlossen, dass die skorbutischen Erscheinungen einfach auf Inanition beruhen.

Sehr interessant sind die Versuche der Autoren, die die Wirkung der sog. Antiskorbutika behandeln. Als solche wurden frische Kartoffeln, Weisskohl, Löwenzahn, Karotten, Zitronensaft, Himbeersaft und Sauerrampfersaft erkannt. Es ist eine sehr interessante Tatsache, dass die verschiedenen Antiskorbutika sich in bezug auf die Stabilität des antiskorbutischen Prinzips wesentlich unterscheiden. Es kommt dies beim Erhitzen, Aufbewahren und Trocknen zum Vorschein. Über den Grund dieser Erscheinungen sind wir vorläufig noch ganz im dunkeln. Wir wissen nicht, ob es sich in diesen Fällen um verschiedene oder um gleiche Substanzen handelt. Es scheint nach den neueren Untersuchungen klar zu sein, dass dies verschiedene Verhalten nicht unbedingt auf das Vorhandensein verschiedener Substanzen zurückzuführen ist. Es ist wohl möglich, dass dies durch verschiedene Bindungsweise und die Reaktion des Saftes bedingt ist.

Man kann im allgemeinen sagen, dass Antiskorbutika gekocht schwächer wirken als im rohen Zustande, ferner schädigt das Erhitzen auf 110—120° dieselben mehr als das Aufkochen bei 100°. Es ist nicht nur das Erhitzen, das schädigend wirkt, sondern auch das Trocknen, was übrigens mit der Erfahrung übereinstimmt, die bei der Ätiologie des menschlichen Skorbutus gemacht worden ist. Dabei scheint die relative Feuchtigkeit des Trockenraumes von Einfluss zu sein, so z. B. geht die antiskorbutische Wirkung von Kartoffeln, Karotten, Löwenzahn und Weisskohl bei Zimmertemperatur schneller verloren als im Brutraum bei 37°. Der getrocknete Löwenzahn ist, zum Unterschied vom Weisskohl, vollständig unwirksam. Anders verhalten sich die Säfte, die aus diesen Gemüsearten gewonnen wurden. So verliert der Weisskohlsaft seine prophylaktischen Eigenschaften, wenn er 10 Minuten lang auf 60°, 70° oder 100° erhitzt wird, dasselbe geschieht, wenn der Saft bei gewöhnlicher Temperatur (unter Zusatz von Antiseptika) oder auch im Eisschrank aufbewahrt wird.

Gänzlich verschieden im Verhalten zu den oben erwähnten Nahrungsmitteln ist der Zitronensaft, und wie wir später sehen werden, auch die Milch. Man kann den Zitronensaft eine Stunde auf 110° erhitzen, ohne dass derselbe merklich an Wirkung verliert. Nun enthält der Saft 7% Zitronensäure, und dies brachte die Autoren auf den Gedanken, dass vielleicht unter Wirkung der Säure die antiskorbutische Substanz stabiler wird. Der Gedanke wurde bestätigt durch den Befund, dass auch andere sauer

reagierende Säfte, wie Himbeer- und Sauerampfersaft dieselbe Stabilität gegen Erhitzen aufweisen. Auch die Hitzestabilität des Weisskohl- und Löwenzahnsaftes lässt sich durch Säurezusatz bedeutend erhöhen, ein Haltbarmachen auf die Dauer gelang aber nicht.

Diese letzte Erfahrung wurde daraufhin bei den Extraktionen nutzbar gemacht. Wurde der frisch getrocknete Weisskohl statt mit reinem Alkohol, mit Alkohol extrahiert, dem etwas Zitronensäure zugesetzt wurde, so erwies sich dieser Extrakt für Meerschweinchen viel wirksamer. Andere Darstellungsverfahren wie das Dialysieren oder Extrahieren mit Petroläther schlugen fehl. Wenn wir noch hinzusetzen, dass es den Autoren nicht gelang, durch intraperitoneale Einspritzungen den experimentellen Skorbut der Meerschweinchen günstig zu beeinflussen, so hätten wir das grosse Tatsachenmaterial besprochen, das Holst und Fröhlich seit vielen Jahren gesammelt haben.

Wie wir daraus ohne weiteres erkennen können, waren diese Angaben für eine chemische Untersuchung nicht gerade ermutigend. Ausser der geringen Stabilität der Substanz waren weitere Schwierigkeiten im Wege. Erstens schienen mir die bekannten Antiskorbutika von keiner so frappanten Wirkung zu sein, wie das von mir untersuchte Vitamin; die nächste Schwierigkeit war, dass die antiskorbutischen Substanzen nur sehr langsam wirken, so dass die Stoffe zu der täglichen Nahrung hinzugesetzt werden müssen, was eine starke Verschwendung des Untersuchungsmaterials bedeuten würde. Trotzdem ging ich an die chemische Isolierung heran. Obwohl die bis jetzt erhaltenen Resultate noch kaum das Problem vorwärts brachten, scheint mir doch, dass dieselben unser Interesse beanspruchen, indem sie uns den Weg für künftige Untersuchungen zeigen. Zuerst galt es, das passendste Rohmaterial ausfindig zu machen. Obwohl der Zitronensaft von Holst und Fröhlich als nicht besonders reich an der antiskorbutischen Substanz gefunden wurde, schien er mir wegen seiner Stabilität als passend für meine Untersuchungen. Ich begann nun nach einem vorläufigen Fraktionierungsversuch, der analog meinen früheren Fraktionierungen aus Reiskleie und Hefe ausgeführt wurde, auf die erhaltenen Fraktionen an Meerschweinchen, die Hafer und Wasser gehalten wurden, zu prüfen. Dabei wurde das Resultat erhalten, dass die Fraktionen, welche bedeutend reicher an dem aktiven Prinzip sein sollten, auch nur eine unbedeutende Wirkung entfalteten. Während z. B. die 11 Kontrolltiere im Durchschnitt nach 24 Tagen eingingen, starben die Meerschweinchen, zu deren Nahrung zersetzter Phosphorwolframsäure-Niederschlag zugesetzt wurde (und zwar in einer Menge, die 300 ccm des Originalsafts entsprechen würde) im Durchschnitt nach 28 Tagen. Dabei wurde beobachtet, dass nach ungefähr 4 Wochen in beiden Fällen die Nahrungsaufnahme vollständig nachliess, so dass meiner Meinung nach der Tod in letzter Instanz auf Hunger zurückzuführen war. Infolgedessen versuchte ich, für diese Experimente Kaninchen zu verwenden, bei welchen die künst-

Ernährung mit grösserem Erfolg angewandt werden kann. Zu meinem grossen Erstaunen sah ich, dass diese Tiere 3 Monate von Hafer und Wasser leben können und erst nach dieser langen Zeit geringe Symptome zeigen.

Darauf ging ich wieder zu den Meerschweinchen zurück und versuchte andere Ausgangsmaterialien. Ich sah, dass ein täglicher Zusatz von frischem Kartoffelsaft die Tiere etwa 36 Tage lang leben lässt, wurde aber der Saft mit eisigem Bleiacetat gereinigt, so starben die Tiere bereits nach 27 Tagen. Man ersieht daraus, dass auch der Kartoffelsaft für eine chemische Unternehmung nicht geeignet ist. Schliesslich nach vielen Versuchen kam ich auf ein Mittel, die ja auch der Meinung der Autoren nach (wir werden dies später bei der Barlow'schen Krankheit sehen) zu den gegen chemische Eingriffe besonders widerstandsfähigen Antiskorbutika gehört. Ich sah, dass ein Zusatz von 50 ccm roher Milch zum Hafer die Tiere 50 Tage lang (der Versuch wurde hier abgebrochen) ohne Gewichtsverlust erhalten konnte, während 100 ccm nicht ganz genügten. Wird nun das Kasein aus der verdünnten Milch durch Essigsäure bei 50° gefällt, das Laktalbumin durch dialysierte Natriumchloridlösung gefällt und dann die Lösung durch Ausfrieren konzentriert, so wirkt sie lange Zeit wirksam, wenn sie im Eisschrank aufbewahrt wird. Wird dagegen die Lösung mit Kaolin enteiweissst, so scheint die Substanz mitgerissen zu werden. Es ist somit wahrscheinlich, dass die Milch, wenn vorsichtig bearbeitet, Hoffnung auf Erfolge verspricht. Diese Unternehmung wird demnächst in grösserem Umfang vom Verfasser in Angriff genommen.

Die chemische Untersuchung des Zitronensaftes.

Wenn auch diese Untersuchung nach dem oben gesagten wohl kaum ein Schlüssel zu der Erkenntnis des Skorbuts liefern kann, wurde trotzdem eine chemische Untersuchung ausgeführt um zu erfahren, ob nicht im Zitronensaft Substanzen sich nachweisen lassen, die dem Typus des Vitamins entsprechen und die etwa als Abbauprodukte des antiskorbutischen Agens betrachtet werden können. In meinen früheren Arbeiten konnte ich zeigen, dass sich im Zitronensaft (l. c. 22, 32) eine Substanz befindet, die antineuritische Eigenschaften besitzt und eine andere (vom Pyrimidintypus), die bei den Polyneuritis erkrankten Tauben das Leben um einige Tage verlängert. Bei der üblichen Fraktionierung wurden einige neue stickstoffhaltige¹⁾ Substanzen nachgewiesen, deren Zusammenhang mit der antiskorbutischen Substanz mir nicht ganz ausgeschlossen erscheint; es wäre nämlich möglich, dass Spaltungsprodukte derselben darstellen.

¹⁾ Der Zitronensaft des Handels enthält nach Untersuchungen von Feder (65) 0,35 % Stickstoff, Heidelbeerensaft 0,011 %, Rauschbeerensaft 0,014 %.

Die Fraktionierung (66) wurde ganz nach der früheren, bei Beriberi beschriebenen Methode ausgeführt und zwar wurde eine ziemlich grosse Menge Ausgangsmaterial (400 l) in Angriff genommen. Trotzdem gelang es nur Spuren dieser Substanzen aufzufinden, so dass diese Untersuchung noch nicht ganz abgeschlossen ist. Immerhin ist es von grossem Interesse, dass auch im Zitronensaft stickstoffhaltige Substanzen aufgefunden wurden, die einige Ähnlichkeit mit denen aus Hefe und Reiskleie dargestellten, besitzen.

In der Purinbasenfraktion wurde vor allem eine Substanz isoliert, die, obwohl sie augenscheinlich zu den Purinen gehört, keine der für diese Substanzen charakteristischen Reaktionen lieferte. Die Substanz schmolz bei 282° , die Analyse ergab die Formel $C_6H_7O_2N_5$.

In der Pyrimidinbasenfraktion wurde eine andere Substanz dargestellt, die bei $188-189^{\circ}$ schmolz und der Formel $C_9H_{18}O_6N_2$ entsprach. Sie besass alle Eigenschaften der letztgenannten Substanzen. Ausserdem konnten noch geringe Mengen von Stoffen erhalten werden, die in Form von Pikraten abgeschieden wurden und wegen der geringen Ausbeute nicht weiter untersucht werden konnten. Schliesslich ist aus der Cholinfraktion eine Base in Form eines Chloroplatinats dargestellt worden. Dieses Doppelsalz schmolz getrocknet bei 220° und entsprach der Formel $(C_8H_{15}O_2N \cdot HCl)_2PtCl_4$. Es ist nicht ausgeschlossen, dass hier ein Pyridinderivat vorliegt. Bei Erhitzen des Salzes entwickelte sich ein eigentümlicher Geruch, so dass es sich hier kaum um ein Trimethylaminderivat handeln könnte. Die Untersuchung wird übrigens weitergeführt.

Schiffberiberi.

Diese Krankheit, die sehr stark an Skorbut erinnert, wurde sehr eingehend von Nocht (67) untersucht. Den Untersuchungen des letzten Autors zufolge bricht die Krankheit aus auf Segelschiffen, die sich auf langen Reisen befinden, wenn der Vorrat an frischen Nahrungsstoffen dem Ende naht. Charakteristische Symptome sind Anästhesie in den Extremitäten, Kurzatmigkeit und schliesslich Tod durch Herzschwäche. Zum Unterschied von tropischer Beriberi erholen sich die Patienten sehr rasch, wenn sie frischen Proviant erhalten. Neuritis-Symptome kamen nur äusserst selten zur Beobachtung. Holst und Fröhlich (68) halten diese Krankheit für sehr nahe mit Skorbut verwandt, es scheint demnach, dass alles, was über Skorbut gesagt wurde, auch für die Ätiologie von Schiffberiberi gilt.

Barlowsche Krankheit (Infantiler Skorbut).

Diese Krankheit kommt wohl ausschliesslich bei künstlich ernährten Kindern vor. Als Ursache der Krankheit darf man jetzt entweder sterilisierte Milch oder Zusatz von den Handelsmehlpräparaten annehmen. Nach

neuesten Statistiken soll die Krankheit (Lane-Claypon 69) äusserst selten vorkommen. Es sind aber früher förmliche Endemien beobachtet worden.

Die erste eingehende Untersuchung dieser Krankheit verdanken wir Thomas Barlow (70). Die pathologischen Veränderungen wurden besonders sorgfältig von Fränkel untersucht (71). Das Krankheitsbild zeigt eine grosse Analogie der Symptome mit dem experimentellen Skorbut des Meerschweinchen; darauf haben Holst und Fröhlich ganz besonders aufmerksam gemacht. Auch die grosse Ähnlichkeit mit dem Skorbut der Erwachsenen brachten Barlow, Holst (l. c. 68) und Looser (72) auf die Idee, dass beide Krankheiten vollständig identisch seien. Diese Annahme findet eine ganz besondere Stütze in der Arbeit von Hart (79). Er fütterte einen jungen Affen (*Macacus*) mit kondensierter Milch und fand dieselben Knochenveränderungen, auch die sonstigen Symptome, wie bei dem erwachsenen Affen mit derselben Diät. In der Arbeit finden wir ausserdem eine vorzügliche und detaillierte Beschreibung der pathologisch-anatomischen Befunde. Dagegen kam Vortisch van Vloten (74) zum Ergebnis, dass, obwohl beide Krankheiten identisch sind, beide als Ursache einen Mangel an Salzen, nämlich Kalium- und Eisensalzen haben.

Durch die Arbeit von Neumann (75), der zuerst auf die Möglichkeit eines kausalen Zusammenhanges zwischen dem Kochen der Milch und dem Entstehen der Krankheit hinwies, ist unser Interesse an diesem Gebiet ganz erheblich gestiegen. Er fand, auf Grund einer ganzen Anzahl von Fällen, dass Kinder, die mit Milch ernährt wurden, die im Soxhlet-Apparat 10 Minuten erhitzt war, an infantilem Skorbut litten. Er glaubte, dass der Grund dafür im Entstehen von toxischen Substanzen durch den Kochprozess zu suchen ist. Neumanns Resultate wurden von Heubner (76) bestätigt, und von A. Meyer (77) mit weiteren Belegen versehen. Einige Fälle, die die Autoren auf gekochte Milch zurückführen, wurden vor kurzer Zeit von Brachi und Carr (78) beschrieben. Die erkrankten Kinder können durch Zusatz von roher Milch oder Zitronen- und Fruchtsäfte vollständig geheilt werden. Wir sehen aus dem Gesagten unzweideutig, dass der infantile Skorbut dieselbe Ätiologie besitzt wie der Skorbut der Erwachsenen. Die beiden Formen sind durch Fehlen einer antiskorbutischen Substanz in der Nahrung bedingt. Da unter Umständen Milch bei Kindern die einzige Nahrung darstellt, so wollen wir vor allem untersuchen, ob die Milch die antiskorbutische Substanz enthält und zweitens ob dieselbe durch Erhitzen zerstört werden kann. Ich glaube, dass die Frage von so grossem praktischem und theoretischem Interesse ist, dass eine erschöpfende Behandlung des Themas am Platze ist.

Wir sahen aus dem unter Skorbut Gesagten, dass Milch ohne jeden Zweifel das antiskorbutische Agens enthält. Ausserdem wurde, wie schon

oben erwähnt (l. c. 32), auch Vitamin darin nachgewiesen. Weiter unten werden wir finden, dass in der Milch die Gegenwart einer Substanz nahezu sicher gestellt wurde, die höchstwahrscheinlich derselben Substanzgruppe angehört, wenn nicht ganz identisch mit der früher behandelten ist und die das Wachstum junger Tiere fördert. Obwohl wir wissen, dass die hier in Frage kommenden Substanzen gegen Erhitzen sehr empfindlich sind, gehört allerdings Milch zu der Reihe der Nahrungsstoffe, in welchen die antiskorbutische Substanz relativ stabil ist. Ist nun Grund vorhanden, in der Milch die Gegenwart einer Substanz anzunehmen, die durch Erhitzen zerstört wird? Zur Beantwortung dieser wichtigen Frage werden wir das vorhandene Tatsachenmaterial in zwei Kapitel teilen, und im ersten die durch Tierexperimente, im zweiten die durch klinische Beobachtung gemachten Erfahrungen behandeln.

Tierversuche über den Unterschied zwischen roher und gekochter Milch.

Diese Untersuchungen können in zwei Teile zerlegt werden, solche mit arteigener und artfremder Milch. In diesen beiden Fällen scheinen die Ergebnisse nicht ganz gleichsinnig zu sein. Die erhaltenen Resultate scheinen übrigens nicht von einer Art zu sein, auf die man weiter bauen könnte. Gerlach (79) fand, dass Kälber, die auf gekochte Kuhmilch gesetzt wurden, nach den ersten 4 Tagen nicht so gut gediehen, als die Kontrollen, die rohe Milch erhielten. Alle Symptome sollten nach Zusatz von Kochsalz zum Schwinden gebracht werden. Sterilisierte Milch wurde dagegen von Price (80), Doane und Price (81) für Kälber als schädlich gefunden (Durchfall). Hier muss eingeschaltet werden, dass wir pasteurisierte (bis 60—70° erhitze), gekochte (nur zum Sieden gebracht und dann weggestellt) und sterilisierte Milch (d. h. solche, die längere Zeit über 100° erhitzt wurde), unterscheiden. H. H. Dean (82) konnte einen Unterschied zwischen roher und pasteurisierter Milch bei Kälbern nicht feststellen. Hittcher (83) konnte bei Experimenten derselben Art, wie die obengenannten, nur unter Zusatz von verschiedenen Salzen, obwohl er die Versuche über längere Zeit ausführte, nicht zu bestimmten Resultaten gelangen.

Experimente mit artfremder Milch an Meerschweinchen.

Bartenstein (84) fand, dass mit gekochter oder roher Kuhmilch ernährte Meerschweinchen einer Krankheit anheimfallen, die er als ähnlich der *Osteotabes infantum* ansieht. Fröhlich (85), der die Versuche von Bartenstein wiederholte, ist ganz derselben Meinung. Er findet ebenfalls, dass auch die rohe Kuhmilch für die Meerschweinchen offenbar keine passende Nahrung ist. Er findet bei den so ernährten Tieren eine auffallende Porosität der Knochen, wurden dagegen die Tiere mit Milch ernährt, die 10—30

Minuten auf 100° oder 1 Stunde auf 112° erhitzt wurde, so bekamen die Tiere eine ausgesprochene, von vielen Frakturen begleitete Knochenbrüchigkeit. Blutungen um die Frakturen kamen nicht zur Beobachtung, auch die Zähne waren nur ausnahmsweise gelockert. Ein Zusatz von Kohl oder Zitronensaft konnte die Knochenbrüchigkeit nicht verhindern. Es ist demnach bewiesen, dass die hier entstehende Krankheit mit Skorbut nicht identisch ist und eher an Rachitis erinnert, auf welches Thema wir noch zu sprechen kommen. Ausserdem unternahm Fröhlich eine Reihe von Versuchen, in welchen die gegen Skorbut schützende Wirkung der Milch an mit Hafer gefütterten Meerschweinchen probiert war. Er kam hier zu folgenden Ergebnissen: Während ein Zusatz von pasteurisierter (auf 70° erhaltener) Milch den Skorbut zu verhindern vermag, verliert die Milch vollständig diese Wirkung, wenn sie 10 Minuten auf 98° erhitzt wird. Moro (86) fand ebenfalls, dass Meerschweinchen mit Kuhmilch nicht gedeihen können.

Mäuse und Ratten.

Keller (87) schliesst aus seinen Versuchen an Mäusen zum Schluss, dass zwischen roher und gekochter Milch kein Unterschied der Ausnutzung besteht. Janet Lane-Claypon (88) fütterte Ratten mit roher und gekochter Milch und konnte keinen Unterschied im Gedeihen derselben wahrnehmen. Da aber in beiden Serien Brot zugesetzt wurde, aus dem die Tiere vielleicht die antiskorbische Substanz schöpfen konnten, so sind die Versuche nicht ganz einwandfrei.

Kaninchen.

Moro (l. c. 86) fand, dass Kaninchen von Kuh- und Frauenmilch nicht leben können, so dass hier ein Vergleich zwischen der rohen und gekochten Milch nicht gemacht werden konnte.

Hunde.

Rodet (89) fand, dass Hunde sehr gut Kuhmilch vertragen. Die Milch wurde roh, kurz aufgekocht und lange gekocht verabreicht. Die kurz gekochte Milch wurde am besten ausgenutzt. Brüning (90) verabreichte gekochte Kuhmilch und rohe und gekochte Ziegenmilch, weshalb ein Vergleich der Resultate nicht möglich ist. In einem anderen Experiment wurden bessere Resultate mit gekochter als mit roher Kuhmilch erhalten, der Versuch dauerte 75 Tage, bloss ein Tier in jeder Serie. Moro (l. c. 86) fand, dass mit Frauenmilch ernährte Hunde sich sehr schlecht entwickeln, dagegen gut mit Kuhmilch. Peiper und Eichloff (79) fütterten Hunde mit roher und sterilisierter Kuhmilch. Diese Tiere litten an hochgradiger Knochenbrüchigkeit. In einem Falle wurde eine kleine Blutung an der Epiphysengrenze aufgefunden.

Schweine und Ziegen.

Brüning (l. c. 90) fütterte junge Schweine mit roher und gekochter Kuhmilch mit natürlich gesäugten Tieren als Kontrolle. Gekochte Kuhmilch wurde besser ausgenutzt als rohe. Bamberg (92) machte den interessanter Vergleich zwischen zwei Milcharten, nämlich zwischen einer keimfreien und gewöhnlichen Marktmilch. Hier konnte gezeigt werden, dass die keimfreie Rohmilch besser ausgenutzt wurde als die entsprechende gekochte, während bei der Marktmilch das Umgekehrte der Fall war. Bei Ziegen konnte Brüning (93 l. c. 90) bessere Ausnutzung der gekochten Kuhmilch finden während Brückler (94) einen grösseren Gewichtsansatz bei roher Milch fand. Esser (95) fand bei Ziegen bei Anwendung stark sterilisierter Milch Knochenveränderungen, wie bei der Barlowschen Krankheit bzw. Osteotabes infantum. Vergleichen wir die hier angeführten Resultate, so müssen wir zugeben, dass die Resultate ziemlich unsicher ausfallen. Sterilisierte Milch scheint aber einen entschieden ungünstigen Einfluss zu besitzen. Was die gekochte Milch anbelangt, so scheint die rohe arteigene der gekochten vorzuziehen zu sein, während bei heterogener Milch das Umgekehrte der Fall ist. Diese Schlussfolgerungen scheinen mir doch zu unsicher, insbesondere, da die Versuchsdauer in den meisten Fällen viel zu kurz ausfiel um irgendwelche bindende Schlüsse daraus ziehen zu können.

Klinische Erfahrungen über den Wert der rohen und gekochten Milch.

Aus der Mitteilung von Neumann (l. c. 75) ersehen wir, dass er die Barlowsche Krankheit auf längerdauerndes Erhitzen der Milch im Soxhlet-schen Apparat zurückführt, derselben Meinung schloss sich auch Heubner (l. c. 76) an. Diese Autoren kommen zu dem Schlusse, dass eine langdauernde Ernährung mit stark und lange erhitzter Milch als die Ursache des infantilen Skorbutus angesehen werden muss. Cassel (96) sprach sich dahin aus, dass gewöhnlich die Barlowsche Krankheit zum Stillstand gebracht werden kann, wenn man die kurz zum Sieden erhitzte Milch sofort den Kindern verabreicht. Doch in manchen Fällen versagte dies Mittel vollständig, welche Erfahrung auch öfters mit pasteurisierter Milch gemacht wurde; nur die Darreichung roher Milch führte in diesen Fällen zur Besserung. Finkelstein (97) spricht sich sehr entschieden gegen das Kochen der Frauenmilch aus, während die nur gekochte Kuhmilch seiner Meinung nach nicht schädlich sein soll. Moro (98) glaubt, dass die Frauenmilch durch Kochen an Nahrungswert verliert. Langstein und L. F. Meyer (99) kamen in ihrem Buch über die Säuglingsernährung zu dem Ergebnis, dass weder die Frauen- noch die Kuhmilch durch das Kochen an Wert verliert. Potpeschnig (100) fand, dass Frauen- und Kuhmilch durch halbstündiges Erhitzen auf 60° nichts von ihrem Wert einbüsst. Mit Kuh- und Ziegenmilch kamen die Autoren

zu sehr verschiedenen Ergebnissen. Was die sterilisierte Kuhmilch betrifft, so kamen Uhlig (101), Variot (102), Koplik (103) zu günstigen Resultaten, während Leeds und Davis (104), Halipré (105) mit sterilisierter Milch und Hohlfield (106) mit gekochter, keine guten Resultate erhielten. Bendix (107) fütterte ältere Kinder mit sterilisierter Milch, aber unter Zusatz von Apfelgelee und Brot, natürlich war hier der Erfolg ein guter. Was die Beziehungen zwischen dem Kochen der Milch und Entstehen von Skorbut und Rachitis betrifft, sind die Autoren auch nicht einig.

Variot (108) sah unter 3000 Kindern, die mit sterilisierter Milch ernährt worden sind, keinen Skorbut und nur einige Rachitisfälle, zu demselben Ergebnis kam auch Bresset (109). Escherich (110) schliesst sich der Meinung der französischen Autoren völlig an, glaubt aber, dass Rachitis unter den künstlich ernährten Kindern öfter vorkommt, im Vergleich mit den brusternährten. La Fetra (111) berichtet über Skorbutfälle bei brusternährten Kindern, wo die Mütter sich in schlechtem Gesundheitszustand befanden. Aus den Fällen von infantiler Beriberi, die von Andrews (l. c. 36) beschrieben wurden, ersehen wir, dass das sehr gut möglich ist, in diesem Falle war nämlich die Milch frei oder arm an Vitamin, weil die Mütter ebenfalls an Beriberi litten. Lust (112) und Carel (113) sahen dagegen Skorbutfälle bei sterilisierter Milch, Plantenga (114) berichtet über Fälle, die unter Ernährung mit Milch entstanden sind, die eine halbe Stunde auf 70° erhitzt worden ist. Schliesslich berichtete Bendix (115) einen Skorbutfall, der durch Kuhmilch entstanden war (es ist nicht angegeben, ob die Milch erhitzt wurde), durch Mehlahrung verschlimmert und durch rohe Milch geheilt wurde. Janet Lane-Claypon (l. c. 69) kam bei der Übersicht eines sehr reichhaltigen Materials eines Säuglingskonsultation-Ambulatoriums zu dem Ergebnis, dass ein kurzes Kochen der Milch weder Skorbut noch Rachitis verursacht.

Bei der kritischen Betrachtung des vorhandenen Materials möchte ich folgende Punkte kurz hervorheben. Die oben besprochenen Arbeiten wurden in einer Zeit ausgeführt, als man noch nichts über das Vorhandensein von Substanzen in der Nahrung wusste, die gegen Erhitzen nicht sehr stabil sind und offenbar eine sehr grosse physiologische Bedeutung besitzen. Insbesondere ist nicht beachtet worden, ob nicht andere Nahrungsstoffe zu der Milchdiät zugesetzt worden sind, wie dies eben sehr oft vorkommt. Bei einer ambulatorischen und Konsultationspraxis ist diese Kontrolle überhaupt nicht ausführbar. Dies schmälert die oben besprochenen Resultate in ihrem Wert sehr erheblich. Auch die Versuchszeit war in den meisten Fällen zu kurz bemessen.

Es ist jetzt kaum mehr möglich daran zu zweifeln, dass die Milch Stoffe enthält, die gegen Beriberi und Skorbut schützen. Andererseits wissen wir, dass diese Substanzen gegen erhöhte Temperaturen sehr instabil sind.

Damit ist es nicht gesagt, dass z. B. ein Erhitzen auf 100° die Substanzen vollständig zerstört. Das Verhältnis der durch Erhitzen zersetzten Substanz zu der ursprünglich vorhandenen hängt offenbar von vielen Umständen ab. Ausser der Temperatur kommt auch die Dauer der Erhitzung und die Reaktion der Flüssigkeit in Betracht. Zuletzt darf auch nicht vergessen werden, dass Vitamine in der Milch nicht unbedingt in konstanter Menge vorkommen müssen, sie können auch ganz fehlen (vgl. Andrews l. c. 36). Der letztere Umstand hängt offenbar mit dem Gehalt der Nahrung an diesen Stoffen zusammen; ist die Nahrung an diesen Substanzen arm, so werden sie in der Milch entweder ganz fehlen oder nur in kleinen Mengen vorkommen. In letzterem Falle wird auch ein mässiges Erhitzen die Milch minderwertig machen. Deswegen müssen wir die Frage, ob das Kochen der Milch Skorbut oder Rachitis verursachen kann, jedenfalls so lange offen lassen, bis die Frage mit neuen Methoden und unter Berücksichtigung der obengenannten Punkte erforscht ist.

Die Frage liesse sich entscheiden, wenn wir eine Methode besässen, die eine quantitative Bestimmung der hier in Betracht kommenden Substanzen gestattete. Da dies bis jetzt leider noch nicht möglich ist, möchte ich folgendes Verfahren vorschlagen. Wir wissen ganz genau, dass diese Substanzen Stickstoff enthalten, man könnte nun den Reststickstoff nach dem Entfernen des Kaseins und des Laktalbumins bestimmen. Das Verfahren würde sich folgendermassen gestalten. Nach der Entfernung des Kaseins bei etwa 37° mit verdünnter Essigsäure, könnte man im Filtrat das Laktalbumin durch Fällen mit dialysierter Eisenlösung beseitigen. Im letzten Filtrat könnte man die vitaminähnlichen Substanzen mit Phosphorwolframsäure fällen. Daraufhin würde man den Niederschlag nach Kjeldahl verbrennen und im Phosphorwolframsäurefiltrat den Allantoinstickstoff noch ausserdem bestimmen. Ich glaube, dass auf diese Weise, bei strenger Innehaltung gleicher Bedingungen, gute Vergleichsresultate erzielt werden können. Ich habe einige solche Bestimmungen ausgeführt und habe gefunden, dass die Werte ziemlich schwankend sind. Ich habe 2 mgr Vitaminstickstoff und 36 mgr Allantoinstickstoff im Liter Rohmilch gefunden. Daraufhin könnte untersucht werden, ob der Stickstoffgehalt in den in Frage kommenden Fraktionen beim Erhitzen abnimmt, und ob die betreffende Milch, mit welcher in dieser Beziehung schlechte klinische Erfahrungen gemacht werden, auch wirklich eine merkliche Abnahme der zur Vitamingruppe gehörenden Substanzen aufweist. Dass bei dieser Methode Resultate zu erwarten sind, zeigt die Arbeit von Bordas und Raczkowski (104), die gefunden haben, dass das Lecithin der Milch durch Kochen eine partielle Zersetzung erleidet, bei 60° soll sogar eine beträchtliche Menge dieser Substanz verschwinden, bei 95° 28%, und wird die Milch 30 Minuten auf 105—110° erhitzt, so soll nicht weniger als 30% des Lecithins verloren gehen. Eine Arbeit dieser Art ist auch neuerdings

ber n. physiol. Bedeutung gewisser bisher unbekannter Nahrungsbestandteile, d. V. Hämmerl. 100

von Stepp (117) unternommen worden. Er fand, dass ein zweitägiges Erhitzen der Mäusenahrung mit Alkohol und Zusetzen des Extraktes, die hier in Betracht kommenden Substanzen vollständig vernichtet, während ein sechsständiges Erhitzen nicht so gefährlich ist (s. S. 173). Erhitzen mit Wasser tut es in geringerem Grade. Stepp gebraucht zwar noch die Bezeichnung Lipide, obwohl es sich hier nicht um Lipide handelt und obwohl das Handelslecithin (Riedel) keinen Effekt auf Mäuse besitzt.

Die Beziehungen zwischen Beriberi und Skorbut.

Zwischen den beiden Krankheiten bestehen zweifellos sehr enge Beziehungen. Kennen wir doch Fälle von Skorbut, wie die von Delpech (118) und Bucquoy (119) während der Belagerung von Paris beschriebenen, die durch ausschliessliche Ernährung mit weissem Reis verursacht waren. Wir sahen, dass dieser Nahrungsstoff bei manchen Tieren, wie bei Hühnern, Tauben, Enten etc. Polyneuritis, bei anderen, wie bei Meerschweinchen und Hunden, dagegen Skorbut hervorruft. Bei Schweinen sollen unter diesen Bedingungen (Holst und Fröhlich l. c. 64) gemischte Symptome von Skorbut und Neuritis auftreten. Holst beschreibt auch Fälle von menschlichem Skorbut, die von sehr deutlichen Neuritisssymptomen begleitet waren. Gouzien (120) beschreibt eine interessante Beriberiepidemie in Hanoï. Es wurde nun den Kranken statt des polierten Reises ein handgemahlener Reis verabreicht, wodurch rasche Genesung der 64 Kranken erzielt wurde. 3 Wochen später kehrten dieselben zur alten Nahrung zurück, diesmal aber erkrankten sie sämtlich an Skorbut.

Wir sahen in früheren Kapiteln, dass das verschiedene Verhalten gewisser Tiere gegenüber dem Fehlen derselben oder nur analoger Substanz in der Nahrung auf die Eigentümlichkeiten des Purinstoffwechsels zu setzen sind, die von einer strengen Spezifität der darauf passenden Fermente begleitet sind. Demnach würde es uns nicht wundern, dass artfremde Milch, jedenfalls für manche Tierarten, so schlechte Resultate liefert. Wir können jetzt vermuten, dass jede Tierart, die auf ihren Stoffwechsel eingestellten Hormonogene aufweist, die wiederum von der dem Tiere angepassten Nahrung abhängig sind.

Trotzdem müssen wir Skorbut und Beriberi als völlig verschiedene Krankheiten auffassen, die beide jedoch durch Fehlen gewisser Substanzen in der Nahrung verursacht sind. Dass hier zwei verschiedene Substanzen in Betracht kommen, dafür sprechen viele Tatsachen. Das Beriberi-vitamin ist ohne jeden Zweifel bei weitem stabiler, als die antiskorbutische Substanz. Verschiedene Nahrungsstoffe, deren Zusatz vor Beriberi und Polyneuritis schützt, wie z. B. Hefe, Hafer und Gerste, sind nicht imstande den Skorbut zu verhüten. Manche Umstände sprechen dafür, dass beim Menschen

eine Nahrung, die nur den antiskorbutischen Stoff enthält, gegen beide Krankheiten schützt, Beriberivitamin aber nur gegen Beriberi. Man hat den Eindruck, dass der weniger stabile antiskorbutische Stoff bei der Zersetzung das Beriberivitamin liefert. Dafür spricht das viel raschere Auftreten der Skorbutfälle, wenn die Provision nicht oft genug erneuert wird. Zu dieser Frage liefert die Arbeit von Fürst (121) einen sehr interessanten Beitrag. Dieser Forscher fand, dass gewisse Körner, wie Hafer (der bekanntlich Beriberivitamin enthält) beim Keimen eine Substanz bildet, die vorher fehlte, und die gegen Skorbut schützt. Werden die Körner eingetrocknet, so verlieren sie gänzlich diese neue Eigenschaft, um bei Anfeuchten sie wieder zu gewinnen. Man hätte den Eindruck, dass in den Körnern das viel stabilere Beriberivitamin in latentem Zustande aufgespeichert ist, dass es aber bei dem vitalen Keimungsprozess durch Fermentwirkung in die antiskorbutische Substanz umgewandelt wird. Man wäre geneigt anzunehmen, dass diese letztere Substanz an den vitalen Prozessen (Wachstum) sogar aktiv teilnimmt. Dies ist natürlich nur eine Vermutung, die bisher durch Tatsachen noch nicht gestützt ist.

Diese Vermutungen könnten durch folgendes Schema veranschaulicht werden:

Beriberivitamin \longrightarrow Pflanzenfermente \longrightarrow antiskorbutische Substanz
 \longleftarrow Tierischer Organismus \longleftarrow

Wir kennen augenblicklich keine Fakten, die mit dem obigen Schema nicht in Einklang gebracht werden können. Leider besitzen wir für diese Auffassung noch keine definitiven Beweise und vorläufig wollen wir damit nur ausdrücken, dass beide Vitamine und beide Krankheiten in engem Zusammenhang miteinander stehen.

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Zum Chemismus des Wachstums.

Die modernen Arbeiten auf dem Gebiete des Stoffwechsels haben dargestellt, dass unter Umständen eine Nahrung, die für erwachsene Tiere jedenfalls für eine Zeitlang ausreichend erscheint, für junge Tiere ungenügend ist. Diese Erfahrung wurde von Osborne, Mendel und Ferry (122) gemacht, als sie Ratten lange Zeit mit isolierten und sehr sorgfältig gereinigten Eiweisskörpern, Fett und Kohlehydraten, unter Zusatz von Salzen fütterten. Es waren dies wohl die ersten Stoffwechselversuche, die mit so sorgfältig gereinigtem Material und über so lange Perioden ausgedehnt, ausgeführt wurden. Obwohl ich aus meiner persönlichen Erfahrung nicht glaube, dass Ratten für diese Zwecke geeignete Versuchstiere sind (den Grund dafür werden wir später sehen), verdienen die Versuche aus manchen Gründen grosses Interesse. Es gelang diesen Autoren erwachsene Ratten sehr lange Zeit, oft über ein Jahr ($\frac{1}{4}$ der Lebensdauer dieser Tiere) am Leben zu erhalten. Dies gelang, wenn als Eiweissquelle, Kasein, Glutinin und Edestin gewählt wurde,

gelang aber nicht mit Eiweisspräparaten, denen eine oder mehrere Aminosäuren fehlen, wie z. B. mit Zein und Gliadin. Nach einer gewissen Zeit gingen die Tiere ein, und zwar ganz plötzlich, resp. sie würden eingegangen sein, wenn nicht gleich eine Diätänderung vorgenommen wurde. Auch junge Tiere konnten eine Zeitlang unter denselben Bedingungen leben, nur zeigten sie insofern ein merkwürdiges Verhalten, als sie das Wachstum ganz einstellten. Das war ja weiter nicht sonderbar, wenn man die Resultate von Stepp (123) in Betracht zieht. Dieser Forscher konnte zeigen, dass Mäuse mit einer Nahrung, die mit Alkohol extrahiert worden ist, nicht leben können. Insbesondere galt dies für Milch. Wurde dagegen den Tieren der alkoholische Extrakt wieder zur Nahrung zugesetzt, so erholten sich die Tiere rasch. In einer zweiten Arbeit überzeugte er sich (124), dass es sich nicht etwa um verloren gegangene Salze handelt. Er konnte feststellen, dass durch die Alkoholextraktion eine Substanz verlustig geht, die offenbar lebenswichtig ist. Wenn er auch von der Lebenswichtigkeit der Lipoide spricht, so meint er damit nur, dass es sich um alkohollösliche Substanz handelt.

Durch Stepps Arbeit angeregt, versuchten nun die amerikanischen Autoren durch einen Zusatz auch das Futter für junge Tiere vollwertig zu gestalten. Es gelang ihnen dies, als sie den Tieren eiweissfreie Milch, d. h. eine Milch, die von Kasein und Laktalbumin befreit war, zusetzten. In dem Falle erlangten die Tiere die normale Grösse. Fast zu gleicher Zeit erschien eine Arbeit von Hopkins (125), der sich seit einigen Jahren mit demselben Thema beschäftigte. Er fütterte ebenfalls Ratten mit Kasein, unter Fett-, Kohlehydrat- und Salzzusatz, und konnte ebenfalls ein Wachstumsstillstand bei jungen Ratten beobachten. Wurde aber den Tieren eine minimale Milchmenge (einige cem) zugesetzt, so erlangten sie die normale Grösse. Besondere Aufmerksamkeit schenkte Hopkins der Reinheit der verfütterten Produkte. Er machte nämlich die wichtige Beobachtung, dass Handelskasein, sowie das verwendete Fett, manchmal das Wachstum bis zu einem geringen Grad gestatteten. Diese Eigenschaft geht vollständig verloren, wenn man die Nahrungsmittel vorher mit Alkohol extrahiert. Hopkins konnte auf diese Weise nicht nur die Resultate von Stepp bestätigen, sondern fand ausserdem, dass Spuren einer Verunreinigung in der Nahrung schon die erhaltenen Resultate unsicher gestalten. Hopkins hat ausserdem das grosse Verdienst, auf die Analogie zwischen der Wachstumshemmung und die Ausfallerscheinungen, die durch eine partielle Unterernährung verursacht sind, hingewiesen zu haben.

In einer weiteren Arbeit (126) gingen Osborne, Mendel und Ferry einen Schritt weiter. Sie gaben den Versuchsratten ein Futtergemisch, das vollständig fettfrei war, und als Zusatz die proteinfreie Milch in Pulverform, die vorher mit Äther extrahiert worden war. Auch in diesem Falle zeigten die Ratten ein normales Wachstum. Nun analysierten sie sorgfältig ihr Präparat von proteinfreier Milch (127) und stellten eine künstliche protein-

freie Milch her, die soweit möglich die Zusammensetzung des natürlichen Produktes nachahmen sollte. Auch mit diesem Präparat erhielten die Autoren ein vollständiges Wachstum. Sie nehmen an, dass die zum Wachstum nötigen Substanzen anorganische Salze sind. Wir werden in der Diskussion der Resultate sehen, dass dieser Schluss kein zwingender ist. Inzwischen ist von denselben Autoren eine neue Arbeit erschienen (128), die die früher erhaltenen Resultate bestätigt, die Autoren geben aber zu, dass mit der künstlichen proteinfreien Milch doch keine so guten Resultate erzielt werden wie mit dem natürlichen Präparat. Es gelang ihnen, beim Zusatz des künstlichen Präparats, Tiere 114 resp. 277 Tage am Leben zu erhalten, doch nach dieser Zeit gingen sie ein, ohne dass die Sektion einen plausiblen Grund dafür erkennen liess, in einem Falle konnte sogar ein Zusatz des natürlichen Präparats, das Tier nicht mehr vom Tode retten.

In bezug auf diese Frage erhielt ich eine private Mitteilung von Hopkins, dass er auch versucht hat die Milch durch ein Präparat zu ersetzen, welches nach den Angaben der amerikanischen Autoren hergestellt war. Er teilte mir mit, dass, wenn die Handelslaktose, die bekanntlich aus Milch hergestellt wird, durch wiederholtes Umkrystallisieren gereinigt ist, die Tiere gänzlich das Wachstum einstellten. Ich habe mich öfters überzeugt, dass Handelslaktose Spuren Stickstoff, offenbar aus der Milch stammend, enthielt und es wäre möglich, dass diese Spuren schon genügen, um das Wachstum auszulösen.

Von den Arbeiten, die hierher gehören, muss auch eine Arbeit von W. Heubner (129) berücksichtigt werden. Hunde, die mit phosphorarmer Nahrung (Reis, Tapioca) ernährt worden sind, stellten ihr Wachstum ein, begannen jedoch nach Zufuhr von Lecithin, dessen Ursprung nicht weiter angegeben wurde, weiter zu wachsen. Hierher gehört auch eine Arbeit von Schäfer (130), der an weissen Ratten arbeitete. Leider wurde hier eine Nahrung gegeben, die von vornherein ausreichend erscheint, nämlich Brot und Milch. Wurde nun den Tieren eine geringe Menge Ovarial- oder Hypophysengewebe zugesetzt, so hatte dieser Zusatz weder einen Einfluss auf das Wachstum, noch auf den Stoffwechsel. Wurde dagegen Schilddrüsengewebe zugesetzt, so wurde ein grosser Effekt auf das Wachstum konstatiert. Aldrich (131) fütterte weisse Ratten mit Hypophyse. Er konnte keinen Einfluss auf das Wachstum feststellen.

Wir wollen nun die Versuche von Osborne, Mendel und Ferry einer kritischen Betrachtung unterziehen. Wir werden es vor allen Dingen, nach dem vorher Gesagten, merkwürdig finden, dass es diesen Autoren gelungen ist, Ratten mit so gut gereinigtem Material so lange am Leben zu erhalten. Nur zwei Erklärungen sind hier möglich: entweder sind Ratten Tiere, deren synthetische Fähigkeiten so gross sind, dass sie sich die nötigen Vitamine selbst bereiten können, oder es enthielt auch in diesem Falle die

Nahrung die nötigen Substanzen als Verunreinigung. Ich habe selbst einige Versuche an Ratten ausgeführt, denen weisser Reis als einzige Nahrung verabreicht wurde. Nun wissen wir, dass polierter Reis eine sehr unvollkommene Nahrung darstellt. Obwohl die Tiere nur ganz wenig Reis zu sich nahmen, etwa 3—5 g täglich, hatten sie nur 15% im Mittel am Gewicht verloren, als nach 45 Tagen der Versuch abgebrochen wurde. Man ersieht daraus, wie zähe Ratten an ihrem Gewicht festhalten, sogar bei sehr geringer Nahrungsaufnahme. Andererseits ist es möglich, dass die Folgen des Mangels an Vitaminen erst viel später zum Vorschein kommen. Dies scheint auch aus den Versuchen von Osborne und Mendel zu folgen. Überhaupt wäre es ein Fehler, aus den Versuchen an Ratten auf andere Tiere, die sich total verschieden verhalten, Schlüsse übertragen zu wollen. Was den anderen Einwand anbetrifft, so wäre es möglich, dass trotz der Sorgfalt, die bekanntlich Osborne bei der Darstellung seiner Pflanzeneiweisspräparate anwendet, dieselben Verunreinigungen in sich doch einschliessen. Ich glaube, dass die Versuche sich viel eindeutiger gestalten würden, wenn die Pflanzenpräparate längere Zeit mit Alkohol extrahiert wären. Was die chemische Natur der Substanz, die das Wachstum anregt, anbetrifft, so liegt meiner Ansicht nach kein Grund vor, dieselbe mit den anorganischen Substanzen zu identifizieren. Ich glaube persönlich, dass es sich um eine Substanz handelt, die, wenn nicht identisch, so doch vollständig analog der antiskorbutischen Substanz sein muss. Gründe für diese Auffassung wird man in dem vorher Gesagten finden.

Das Wachstum bei Pflanzen.

Bei Pflanzen wurden den oben angeführten analogen Arbeiten wenigstens meines Wissens nach, nur in kleiner Anzahl ausgeführt. Ich konnte nur einige Arbeiten finden, die das Thema behandeln. Doch glaube ich, dass gerade bei Pflanzen die Wachstumsversuche mit einigem Vorteil in Angriff genommen werden könnten. Die Versuche gestalten sich vor allem viel eindeutiger, auch sind es Pflanzen, die uns die hypothetische Wachstumssubstanz liefern. Es wäre deswegen wichtig, die Wirkung der Substanz auf die dazu spezifischen Zellen zu untersuchen.

Macalister (132) untersuchte die wundheilende Wirkung von *Symphytum officinale*. Er konnte aus dem Rhizom der Pflanze Allantoin isolieren, das seiner Meinung nach das wirksame Prinzip der Pflanze darstellt. Er führte nun Versuche an Hyazinthen aus, zuerst stellte er die Zwiebel ins Wasser und setzte dem Wasser Allantoin zu. In diesem Falle wurde gefunden, dass die Wurzeln viel schwächer wuchsen, und zwar wurde die Wachstumshemmung proportional der Allantoinkonzentration gefunden. Da wohl normalerweise kein Allantoin ausserhalb der Zwiebel in der Natur vorkommt, wurde ein anderes Verfahren eingeschlagen. In die Zwiebel wurde nämlich

Allantoinlösung injiziert, indem als Kontrollen andere Zwiebeln mit demselben Wasservolum behandelt wurden. Bei allantoingespritzten Zwiebeln wurde ein viel stärkeres Wachstum und ein schnelleres Blühen konstatiert. Ich habe die Versuche wiederholt, bis jetzt ist es mir nicht gelungen, die Resultate von Macalister zu bestätigen.

Auf Veranlassung von Macalister wurde von Copp in (133) eine ähnliche Untersuchung ausgeführt. Während schwache Lösungen von humussaurem Natrium, apfelsaurem, harnsaurem und oxalsaurem Natrium das Wachstum der Zwiebel förderten und zwar das der Wurzel mehr als das der Blätter, wurden stärkere Lösungen hemmend gefunden. Zellteilung wurde durch Kaffein, humussaures, oxalsaures, ölsaures und oleinsaures Natrium angeregt. Ransom (134) fand dagegen, dass Kaffein die Entwicklung der Samen erheblich hemmt.

Die Versuche über die Wachstumssubstanz sind deshalb so wichtig, weil sie uns vielleicht eine Einsicht in das Wachstum der Tumoren gestatten werden, doch kommen wir darauf im nächsten Kapitel zu sprechen.

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Wachstum und Krebsproblem.

Aus dem vorigen Kapitel erschen wir, dass beim Wachstum junger Tiere die Einwirkung einer Substanz angenommen werden muss, die wir als Wachstumssubstanz bezeichnen wollen. Die Ergebnisse der experimentellen Tumorforschung machen es sehr wahrscheinlich, dass auch ähnliche, wenn nicht identische, chemische Kräfte beim Wachstum der Tumoren eine Rolle spielen können. Damit soll nicht etwa gesagt werden, dass dadurch die Entstehung derselben klargelegt wird. Das Wachstum der Tumoren und die Beeinflussung derselben ist eine Frage von hervorragendem Interesse und ist auch bereits experimentell in Angriff genommen worden. Den ersten Anlass für die experimentelle Entwicklung der vorliegenden Frage hat wohl Paul Ehrlich (135) mit seiner Theorie der athreptischen Immunität gegeben. Dieser Forscher unterscheidet bei den bösartigen Geschwülsten 1. die natürliche Immunität. 2. Immunisierung mit weniger virulenten Tumoren, die vor stärker virulenten Karzinom- und Sarkomarten schützt, wobei sich mitunter der Charakter des Tumors (Karzinom) in das weniger virulente (Adenom) ändert. 3. Immunisierung mit normalem Gewebe: Bashford, Murray und Cramer (136) benutzten zu dem Zweck das Blut normaler Tiere, Schöne (137) Extrakte aus den Embryonen, Borrel (138) und Bridré (139) Milz und Lebergewebe. 4. Athreptische Immunität. Wird ein Mausarkom auf Ratten geimpft, so wächst anfänglich der Tumor ganz normal, nach 8–10 Tagen wird er aber resorbiert. Ehrlich betrachtet diesen Vorgang als eine Erschöpfung der zur Ernährung des Tumors nötigen unbekannten Substanz. Ist das Versuchstier Träger eines grossen Tumors, so ergibt eine zweite Impfung entweder einen ganz langsam wachsenden oder gar keinen Tumor. Die Impfung mit einem sehr virulenten Tumor schliesst meistens eine solche mit einem weniger virulenten vollständig aus. Die Impfung bei graviden Tieren verläuft nach Haaland (140) entweder negativ oder sehr schwach. Die hier angeführte Immunität sowie die sehr verschiedene Virulenz der experimentellen Tumoren führt Ehrlich auf die verschiedene Viridität der Tumoren zu einer unbekannten Substanz zurück. Die Ideen Ehrlichs haben eine Fülle von experimentellen Arbeiten und Kontroversen ausgelöst, auf die jedoch nicht ausführlich eingegangen werden soll. Wir wollen hier nur aufführen, dass Bashford (141) die Ehrlich'sche Theorie als nicht das Wesen der Tumoren erklärend bezeichnet, er weist darauf hin, dass die einzelnen Tumorenstämme sowie die Tumorenträger individuelle Unterschiede aufweisen, die die experimentellen Resultate unsicher machen. Ausserdem macht er darauf aufmerksam, dass Impfungen bei jungen Tieren bessere Resultate geben, dies steht im Widerspruch mit der Erfahrung, die bei menschlichen spontanen Geschwülsten gemacht worden ist. Russel (142) lieferte experimentelle Belege für die Bashfordsche Auffassung. Er fand,

dass es Tumorarten gibt, die Mäuse gegen andere Tumoren immunisieren, andere wieder, wenn operativ entfernt, lassen keine Immunität für denselben Tumor zurück, dagegen immunisieren sie sehr oft gegen andere Tumorarten. Manche stark wachsende Neubildungen geben keine Immunität gegen andere Tumorarten. sie lassen sich auch kaum durch aktive Immunisierung in ihrem Wachstum beeinflussen. Die gleichzeitige Inokulation eines Sarkoms und Karzinoms schwächt meistens das Wachstum des Karzinoms ab.

Vom Standpunkt der Ehrlich'schen Theorie gingen auch diejenigen Arbeiten aus, die sich mit der Empfindlichkeit der unterernährten Tiere gegen Tumorenimpfung befassten. Obwohl eine solche Auffassung allgemein verbreitet war, fand Rous (143), dass Mäuse, die mit Hafer- und Roggenmehl, Mais, Milch und Zucker ernährt wurden und zwar in einer Menge, die gerade zum Erhalten des Lebens genügte, sich verschieden verhielten, je nachdem die Tumoringpfung während der Unterernährung oder vorher vorgenommen wurde. Bei Tieren, die schon grosse Geschwülste aufweisen, hat die Einschränkung der Diät auf das Wachstum der Neubildung gar keinen Einfluss, wird aber diese Einschränkung 4 Tage nach der Impfung vorgenommen so ist das Wachstum der Neubildungen etwas verlangsamt. Dies stimmt auch mit der Erfahrung beim Menschen überein, bei welchem sogar bei einer weitgehenden Kachexie ein energisches Tumorstadium beobachtet wird. Hier muss aber ausdrücklich betont werden, dass eine allgemeine Unterernährung, bei einer sonst kompletten Diät nicht unbedingt ein Fehlen der Wachstumssubstanz mit sich bringen muss. Ausser der athreptischen Theorie, die die Wachstumshemmung auf ein Fehlen einer spezifischen Nahrung (Wachstumssubstanz) zurückführt, gibt es eine andere Theorie, die besonders von H. C. Ross und Cropper (144) verfochten wird, die als Ursache des Krebses die Einwirkung chemischer Substanzen annehmen, die sie unter dem Namen Auxethica gruppieren. Diese Autoren studieren das Phänomen bei den Leukozyten, unter Anwendung einer Gelatineeinbettung, der sie die zu untersuchende Substanzen zusetzen. Sie fanden, dass besonders die Purinsubstanzen und ihre Derivate das Vermögen besitzen die Teilung der Leukozyten hervorzurufen. Ähnliche Substanzen konnten sie auch im Teer und Russ nachweisen, die ja bekanntlich die damit arbeitenden Leute zum Krebs prädisponieren. Auf diese Ideen gestützt injizierte Bayon (145) Kaninchen Teer aus Gasanstalten und erzeugte dadurch epitheliale Wucherungen, über deren Natur er sich nicht weiter ausspricht. In diesem Zusammenhang muss erwähnt werden, dass Rous (146) vor einigen Jahren bei Hühnern ein transplantables Sarkom auffand, das zum Unterschied von den bekannten Tumoren ein abweichendes Verhalten aufweisen soll. Dies Sarkom, das am Anfang sehr wenig infektiös war und sich nur bei Hühnern einer Rasse (Plymouth Rocks) weiterkultivieren liess, wurde allmählich durch Weiterimpfung eininfektöser, eine Beobachtung, die übrigens auch bei anderen

Tumoren gemacht wurde. Schliesslich wurde der Tumor so hochgradig virulent, dass sogar das getrocknete Gewebe, sowie das Berkefeld-Filtrat eines Extraktes desselben, besonders bei jungen Tieren Tumoren erzeugte, die sich weiter impfen liessen. Das letztere allerdings nur dann, wenn der Berkefeld-Filter defekt war. Obwohl dieser Tumor jetzt allgemein als infektiöser Natur (Filterpasser) betrachtet wird, darf nicht ausser acht gelassen werden, worauf Rous (147) selbst hinweist, dass es sich auch um eine chemische Substanz handeln kann. Das Hühnersarkom lässt sich nach Angaben von Murphy und Rous (148) auch in die Eier injizieren, die schon einen Embryo enthalten. In diesem Falle ist der Tumor sogar viel virulenter, was für unsere Auffassung spricht. Der Tumor wächst auch auf Tauben- und Entenembryonen. Interessant ist auch die grosse Analogie zwischen dem Wachstum der Tumoren und der wachstumfähigen Gewebe, wie Embryonen, auf die Rous (149) hinwies zu beobachten. Die Embryonen liessen sich auf Mäuse impfen, wie die Tumoren, auch konnte eine Immunität gegen das embryonale Gewebe erzeugt werden, die offenbar darauf beruht, dass ein Zusammenhang (Gefässe) zwischen dem eingepflanzten Gewebe und dem Wirt fehlte. Höchst interessant ist es, dass die embryonalen Gewebe bei trächtigen Mäusen kein Wachstum zeigten.

Das oben Mitgeteilte lässt die Vermutung berechtigt erscheinen, dass die Tumoren zu ihrem Wachstum einer spezifischen Substanz bedürfen. Dagegen lassen sich für die Auffassung, dass die Ursache des Krebses in einer Stimulation durch eine chemische Substanz besteht, vorläufig noch keine Anhaltspunkte finden. Obwohl diese Auffassung nicht so glatt von der Hand zu weisen ist, lässt sie sich mit der Immunisierung gegen Tumoren kaum in Einklang bringen.

Wollen wir die erste Vermutung als möglich akzeptieren, so müssen wir uns fragen, ob es einen Weg gibt, das Problem experimentell zu lösen. Unserer Meinung nach besitzen wir hier zwei Wege. Als erster Weg erscheint uns die Kultivierung der Gewebe in vitro nach der Methode von Carrel, der zweite in der weiteren Verfolgung der Ideen, die sich an die Wachstumsversuche von Hopkins und von Osborne, Mendel und Ferry anknüpfen, die wir im vorhergehenden Kapitel ausführlich besprochen haben.

Carrel'sche Versuche (150) haben gezeigt, dass sich verschiedene Gewebe in vitro nicht nur halten lassen, sondern auch zum Wachstum gebracht werden können. Auf diese Weise wurden verschiedene Organe und Tumoren über drei Monate lang kultiviert. Hier haben wir ein Mittel in der Hand, um die Wachstumsbedingungen experimentell zu untersuchen. Kurz zusammengefasst ist die Carrel'sche Technik die folgende. Das aseptisch entnommene Gewebe (Carrel 151), wird in ein Plasma mit $\frac{1}{4}$ — $\frac{2}{5}$ destilliertem Wasser gelegt. Am besten eignet sich dazu das Plasma des Versuchstieres selbst oder eines homologen. Jede 3—4 Tage wird die Gewebeskultur 1—2 (Carrel 152) Minuten in Ringerscher Lösung gewaschen und in frisches Plasma

übertragen. Manche Gewebskulturen leben schon $4\frac{1}{2}$ Monate, nach 48 Passagen. Ein Herzfragment pulsiert noch nach 104 Tagen. In künstlichen Medien (Lockes Lösung, Agar und Bouillon) wachsen nur embryonale Gewebe und Tumoren. Bei der Kultivierung der Sarkome von Rous fanden Carrel und Burrows (153) folgende interessante Tatsachen. Dies Sarkom wächst im Plasma desselben Tieres sehr gut, während im Plasma sarkomatöser Tiere der Tumor nur sehr spärlich wächst. Das normale Plasma lässt sich viel wirksamer machen durch einen Zusatz von Sarkomextrakt. Die normalen Gewebe wachsen im Plasma sarkomatöser Tiere oder beim Zusatz von Sarkomextrakt ebenfalls besser. Zu normalem Plasma wurde auch öfters Muskelextrakt oder Extrakt aus embryonalem Gewebe zugesetzt, doch wurde auf den Effekt dieser Zusätze nicht speziell eingegangen. Die Carrel'schen Versuche sprechen deutlich für die von mir vertretene Auffassung, und wir kommen jetzt zu der Besprechung des zweiten von uns gegebenen experimentellen Weges.

Dieser Weg besteht in der experimentellen Untersuchung der Bedingungen, unter welchen das normale Wachstum der Tiere vor sich geht. Durch Wahl einer bestimmten Diät, über welche wir im vorigen Kapitel berichtet haben, gelingt es das Wachstum der Tiere einzuschränken. Vor allem muss untersucht werden, ob solche Tiere bei einer kompletten Diät, aber ohne Wachstumssubstanz, sich mit Tumoren impfen lassen. Ist das der Fall, so muss daraus geschlossen werden, dass der Tumor zum Wachstum einer anderen Substanz bedarf, als der Organismus selbst. Beim negativen Verlauf dieses Experiments könnte vielleicht der Tumor zum Wachsen gebracht werden, wenn den Tieren ein alkoholischer Extrakt des betreffenden Tumors zu der Nahrung zugesetzt wird. Ist unsere Voraussetzung richtig, so drängt sich eine ganze Anzahl von Problemen auf; es könnte zum Beispiel untersucht werden, ob durch Tumorextrakt, beim Fehlen der Wachstumssubstanz, normales Wachstum wieder herbeigeführt werden kann. Eine ganze Anzahl dieser neuen Fragestellungen ist von mir bereits experimentell in Angriff genommen worden. Es gilt vor allem hier zu entscheiden, ob das normale Wachstum und das Wachstum der Geschwülste durch dieselbe oder durch differente spezifische Substanzen bedingt wird.

Um das Vorhergehende kurz zusammenzufassen, scheint mir die Annahme, dass das Wachstum der Tumoren durch eine chemische Substanz reguliert wird, recht plausibel. Ich nehme an, dass die Wachstumssubstanz, die bei jungen Individuen, beim normalen Wachstumsprozess Verwendung findet, bei Erwachsenen keine Funktion mehr ausübt und abgebaut wird. Da alle diese Substanzen der Nukleingruppe im weitesten Sinne des Wortes zuzählen sind, so wäre es möglich, speziell beim Menschen, dass in der Lebensperiode, in welcher der Nukleinstoffwechsel verlangsamt wird, diese Substanz

nicht mehr abgebaut wird und gewisse Zellen, die aus noch unbekannten Gründen wachstumsfähig sind, zum Wachstum stimuliert.

Auf diesem Wege eröffnet sich auch eine Frage von praktischer Bedeutung; ob es nämlich möglich wäre, durch Eliminierung der Wachstums-
substanz aus der Nahrung das Wachstum des Tumors beim Menschen hintanzuhalten.

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Krankheiten. bei denen die Vitamine eine Rolle spielen könnten.

Die Tatsache, dass die Vitamine eine so bedeutende und trotzdem noch unbekannte physiologische Rolle ausüben, brachte mich auf die Idee, unsere Kenntnisse auch auf pathologische Zustände zu übertragen. Es ist mir dabei bewusst, dass es sich zum Teil um Hypothesen handelt, die nur durch wenige oder sogar gar keine Fakten unterstützt sind. Zu meiner Entschuldigung mag gelten, dass es sich um Krankheiten handelt, die obwohl schon jahrelang in Untersuchung, noch ihrer Lösung harren.

Pellagra.

Dies ist eine Krankheit, die schon wegen ihrer grossen Verbreitung unser Interesse in hohem Masse beansprucht. Diese Volkskrankheit, die schon in Italien unter dem Namen „Italienischer Skorbut“ im achtzehnten Jahrhundert bekannt war, kommt in vielen südländischen Distrikten vor, wo die Hauptnahrung der Einwohner aus Mais besteht. Wir finden sie über Italien, Rumänien, Österreich, Spanien, Portugal, Ägypten, Algier, Vereinigte Staaten, Mexiko und Zentralamerika verbreitet, überall dem Maiskonsum folgend.

Über die Ätiologie dieser Krankheit wissen wir noch sehr wenig positives. Jeder der Autoren arbeitet seiner Idee gemäss, ohne sich um die anderen zu kümmern.

Wenn man die Geschichte dieser interessanten Krankheit liest, sieht man, dass auch hier die Ideen denselben Weg, wie bei der Erforschung der Beriberi gingen. Zuerst dachte man an eine Intoxikation, dann an eine Infektion. Als die diesbezüglichen Forschungen nicht zum Ziele führten, musste man neue Wege suchen. Dies führte unter anderen zur photochemischen Theorie, die sich momentan in voller Blüte befindet. Es ist jedoch sehr unwahrscheinlich (die Gründe werden wir gleich sehen), dass diese Theorie uns zum Ziele führen wird. Man muss es aber merkwürdig finden, dass die interessanten neuen Ergebnisse der Beriberiforschung auf die Pellagrauntersuchung, keinen Einfluss ausgeübt haben. Meine dahin zielenden Äusserungen (l. c. 22) blieben vorläufig ohne Wiederhall.

Die Krankheit tritt in der Regel im Frühling und im Herbst auf, mit Erythem, später Sklerose der Haut an unbedeckten Hautteilen, wie an Gesicht, Händen und Füssen. Ferner entsteht Diarrhöe, sowie nervöse Symptome, mit psychischer Depression, die mitunter zum Selbstmord führt. Der ganze Ernährungszustand der Kranken scheint schwer zu leiden, eine allgemeine Kachexie wird oft beobachtet. Pathologisch-anatomisch wurden u. a. Entzündungsherde in der grauen Substanz des Grosshirns, sowie Sklerose des Rückenmarks gefunden.

Bei der Durchsicht der einschlägigen Literatur finden wir 5 verschiedene Theorien, die alle auch jetzt noch ihre Anhänger finden. Das sind die Intoxikations-, die Autointoxikations-, Infektions-, die photodynamische und die partielle Unterernährungstheorie. Alle diese Theorien werden hier nacheinander ihre Besprechung finden. Ausserdem finden wir Meinungen, wie die von Hodson (154) dass Pellagra überhaupt keine Krankheit mit einheitlichem Charakter darstellt.

Die Intoxikationstheorie.

Diese Theorie ist seit Jahren in Italien adoptiert und findet jetzt noch viele Anhänger. Sie wurde wohl zuerst von Ceni (155) und Otto (156) ausgesprochen. Sie machten die Beobachtung, dass gewisse *Aspergillus*- und *Penicillium*-Arten, die konstant in verdorbenem Mais aufgefunden werden, toxische Substanzen produzieren. Diese Substanzen, isoliert und Tieren eingeführt, lösen Symptome aus, die der Pellagra ähnlich sein sollen. Diese Theorie wurde von Lombroso (157) erweitert, mit Belegen versehen und energisch verteidigt. Er fand dann Anhänger in Gosio (158) und Gavina (159). Lombroso konnte aus diesen Schimmelpilzen ein Alkaloid isolieren, das Tieren einverleibt toxische Symptome hervorrief, welche etwa an *Ergotismus* erinnern sollten. Er fasste die Krankheit auf als eine chronische Toxämie, durch toxische Substanzen verursacht, welche im Mais unter der Wirkung von Schimmelpilzen entstehen sollen. Diese Theorie ist auch jetzt in Italien, mit geringen Abänderungen, akzeptiert, wie die Arbeiten von Bertarelli (160), Antonini (161) und neuerdings von Camurri (162) bezeugen. Der letzte Forscher glaubt, dass ausser der Unzulänglichkeit des Maises als Nahrung und Mangel an Nährsalzen, noch eine Intoxikation eine Rolle spielt. Er macht darauf aufmerksam, dass Maisfermente, die seiner Meinung nach auch die toxischen Produkte zu bilden imstande sind, noch nach dem Kochen ihre Wirkung entfalten können. Auf diese Ideen gestützt unternahm die italienische Regierung eine ganze Reihe von Massnahmen, die zur Verhütung der Krankheit dienen sollten. So wurden spezielle Krankenhäuser für Pellagrakranke eingerichtet, die Einfuhr des verdorbenen Maises wurde verboten, die Bevölkerung wurde mit Trockenapparaten versorgt, die die Verschimmelung verhüten sollten. Die Ansichten über den Erfolg dieser Massnahmen sind geteilt, sie scheinen aber doch wenig Erfolg zu haben, da die Zahl der Kranken angeblich im steten Wachsen begriffen ist.

Die Autointoxikationstheorie.

Sie wurde von v. Neusser (163) und von de Giaxa (164) ausgesprochen und besagt, dass Pellagra durch toxische Produkte verursacht ist, die unter dem Einfluss von Bakterien, speziell *B. coli* im Darm gebildet werden.

Die Infektionstheorie.

Eine grosse Zahl von Mikroorganismen wurde beschuldigt die Ursache der Pellagra zu bilden. Nur wenige davon, nämlich die sich auf experimentelle Belege stützen, können hier ihre Berücksichtigung finden. Di Pietro (165) fand, dass eine Abart von *Penicillium glaucum* die Ursache der Krankheit sei. Ceni (166) isolierte aus den Organen der Pellagrapatienten *Aspergillus fluorescens* und *A. fumigatus*. Tizzoni (167) und Tizzoni und Panichi (168) beschrieben einen Bazillus, den sie *Streptobacillus pellagrae* nennen und der aus den Organen und Fäzes von Pellagrakranken isoliert werden konnte. Auch in verdorbenem Mais konnte er angeblich nachgewiesen werden. Eine Kultur dieses Mikrobs, Meerschweinchen einverleibt, war aber nicht imstande krankhafte Symptome auszulösen, wenn die Tiere nicht auf Maisdiät gesetzt wurden. Sambon (169) kam nach einer sorgfältigen Untersuchung der italienischen Pellagra zum Schluss, dass dieselbe nicht unbedingt an den Genuss von Mais gebunden ist. Die topographische Verteilung der Krankheit (in der Nähe von strömendem Wasser) legte ihm den Gedanken nahe, dass es sich hier um ein Protozoon handelt, das durch eine Fliege von *Simulium*-art übertragen wird. Es gelang ihm aber nicht, das Protozoon nachzuweisen. Ähnliche Assoziation mit der *Simulium*-Fliege wurde auch für Georgia von Roberts (170) vermutet. Neuerdings wurden auch in England Pellagrafälle von Sambon und Chalmers (171) beschrieben und auf dieselbe Ursache zurückgeführt. Die Sambonsche Hypothese fand bis jetzt keine Bestätigung. Auch durch Übertragen von *Simulium*-Fliegen von Pellagrakranken auf Affen konnten keine Krankheitssymptome ausgelöst werden (Hunter 172). Weiss (173) glaubt, dass diese Hypothese unwahrscheinlich ist, da in Südtirol durch Einschränkung des Maisgenusses allein, die Pellagrafälle sehr selten wurden.

Ravitsch (174) glaubt, dass es sich um ein Trypanosom handelt, das durch wandernde Vögel übertragen wird. Vor kurzer Zeit wurde ein merkwürdiges Gebilde aus der Cerebrospinalflüssigkeit der Pellagrakranken von der Englischen Pellagra-Kommission isoliert und beschrieben (175). Aus den guten Erfolgen, die vor vielen Jahren mit Arsenik gemacht wurden und in letzter Zeit mit Atoxyl, und die von Babes und Vasiliu (176) und Babes, Vasiliu und Gheorghus (177) mitgeteilt wurden, wurde auch geschlossen, dass Pellagra eine Infektionskrankheit und zwar durch ein Protozoon verursacht ist. Dieser Schluss ist willkürlich, und andererseits ist Salvarsan nach Cranston (178) und Fritz (179), im Gegensatz zu den früheren Behauptungen, ohne Wirkung bei Pellagra. Auch die Versuche, spezifische Stoffe (Antikörper) im Serum der Patienten nachzuweisen, schlugen fehl. So berichteten Babes und Busila (180), dass es ihnen nicht gelungen sei, spezifische Beziehungen zwischen dem Serum der Kranken und den

Mikroorganismen, die aus den Organen und Fäzes der Kranken gewonnen wurden, festzustellen. Negative Resultate wurden auch mit Extrakten aus Mais erhalten. Tizzoni (181) teilte in einer vorläufigen Mitteilung mit, dass es ihm gelungen sei, ein Präzipitin im Serum der Kranken (mit *Streptococcus pellagrae*) nachzuweisen. Zum Schluss glaubt Alessandrini (182) ebenfalls einen Zusammenhang zwischen Pellagra und fliessendem Wasser entdeckt zu haben, worin Nematodenlarven gefunden worden sind, er glaubt somit die letzteren als Grund des Übels annehmen zu müssen.

Nevius-Hyde (183) kritisiert das ganze Tatsachenmaterial und kommt zum Schluss, dass kein Grund vorliegt, als Ursache der Pellagra Infektion anzunehmen. Er glaubt, dass jedes verdorbene Mehl die Krankheit auslösen kann. In einem Amerikanischen Bericht kamen Clarke, Hamill, Pollock, Curtis und Dick (184) zu demselben negativen Ergebnis. Sie waren nicht imstande Affen mit dem Blute der Pellagrakranken zu infizieren. Casa-Bianchi und Vallardi (185) glauben, dass es sich bei Pellagra um eine Überempfindlichkeit gegen Mais handelt. Sie fanden, dass alle Mais-extrakte toxisch wirken, die wässerigen stärker wie die alkoholischen und ätherischen. Bei maisernährten Tieren liess sich eine Überempfindlichkeit gegenüber den Maisextrakten feststellen. Extrakte aus verdorbenem Mais waren tödlich: Meerschweinchen lassen sich mit Mais nicht erhalten, man findet Läsionen des Gastrointestinal-Traktus, der Niere und Haarausfall. Verdorbener Mais wird von diesen Tieren besser ausgenutzt.

Photodynamische Theorie.

Diese interessante Theorie wurde fast gleichzeitig und unabhängig von einander von Raubitschek, Horbaczewski und Lode publiziert. Wir werden zuerst die Arbeit von Raubitschek besprechen, welche eine wertvolle Kritik der früheren Arbeiten enthält. Raubitschek (183) bespricht zuerst alle Pellagratheorien und findet, dass keine von den ihnen einer eingehenden experimentellen Prüfung standhält. Er findet, dass Polenta (eine aus Mais bereite Speise) immer steril ist, ebenso verlief die Blutuntersuchung der Pellagrakranken immer negativ. Eine bakteriologische Untersuchung der Organe gab auch keine Anhaltspunkte für die infektiöse Natur der Pellagra, und die serologische Untersuchung des Blutes war nicht imstande, Antikörper weder gegen die Proteine des Mais, noch gegen die darin aufgefundenen Organismen zum Vorschein zu bringen. Im Gegensatz zu vielen italienischen Autoren fand Raubitschek, dass Extrakte, von verdorbenem Mais bereitet und Tieren inokuliert, keine Symptome hervorrufen, die man für analog dem Pellagrabild halten könnte.

Auf die Versuche Aschoffs (187) gestützt, der die Gegenwart von Lichtsensibilisatoren in den Lipoiden der Nahrung annimmt, glaubte Raubitschek,

die Ursache der Pellagra darin gefunden zu haben, dass verdorbener Mais toxische Produkte im Organismus entwickelt, die die Fähigkeit besitzen, die Haut gegen Sonnenstrahlen empfindlich zu machen. Es kann wohl als erwiesen gelten, dass solche Substanzen tatsächlich existieren. Konnte doch Hausmann (188) zeigen, dass weisse Mäuse, mit Hämatoporphyrin subkutan injiziert, im strahlenden Licht unter stürmischen Symptomen rasch zugrunde gehen, während die im Dunkeln gehaltenen Tiere keinerlei Krankheitserscheinungen zeigen. Es handelt sich in diesem Falle um eine photobiologische Sensibilisation, die allen fluoreszierenden Körpern zuzukommen scheint. Hausmanns Versuche wurden mit einigen Blutfarbstoff-Präparaten von Hans Fischer und Meyer-Betz (189) wiederholt und vollständig bestätigt. Da hier chemisch reine Präparate zur Anwendung kamen, so ist es interessant, auf dieselben etwas näher einzugehen. Weisse Mäuse, mit reinem Hämatoporphyrin injiziert und mit einer elektrischen Bogenlampe bestrahlt, beginnen nach kurzer Zeitlichtscheu und unruhig zu werden, sich zu beißen und zu kratzen; nach einigen Stunden tritt der Tod ein. Bei den Tieren wurden auch gewisse Veränderungen festgestellt. Die Ohren waren gerötet, die Gallenblase stark mit einem fluoreszierenden Inhalt gefüllt, der das Hämatoporphyrin-Spektrum gab, und ebenso das Duodenum. Dieselben Resultate wurden mit unreinem Mesoporphyrin und dem durch Reduktion und darauffolgende Oxydation an der Luft aus Hämin dargestellten Porphyrin erhalten. Zum Unterschiede von unreinem Mesoporphyrin gab das reine Produkt keine Sensibilisation. Man ersieht daraus, dass diese Lichtwirkung an die Gegenwart spezifisch wirkender Substanzen gebunden ist. Alle Dunkelkontrollen blieben ohne Krankheitserscheinungen am Leben. Das Mesoporphyrin scheint ausserdem auch toxisch zu wirken. Diese Erscheinungen scheinen grosse Ähnlichkeit mit einer Krankheit zu besitzen, die Fagopyrismus genannt wird und beim weissen Rind ausbricht, das mit Buchweizen ernährt wird. Raubitscheks Experimente ergaben folgende Resultate: Weisse Mäuse, mit Mais gefüttert und dem Tageslicht ausgesetzt, begannen nach 4 Wochen stark an Gewicht zu verlieren, nach 6 bis 8 Wochen gingen sie ein, oft unter Krämpfen und mit starker Rötung der Ohren und der Schnauze. Weisse Mäuse, die mit derselben Nahrung im Dunkeln gehalten wurden, ebenso weisse Mäuse, die bei gemischter Diät im Licht, und dunkelgefärbte Tiere (Maisnahrung) im Lichte zeigten keine Krankheitssymptome. Raubitschek fand ferner, dass die sensibilisierende Substanz sich in der alkohol-löslichen Fraktion des Maises befindet. Mais, der vorher einer Alkohol-extraktion unterworfen wurde, war ohne Wirkung auf weisse Mäuse im Lichte, während der Extrakt die toxische Substanz enthielt. Dieselben Resultate wurden mit verdorbenem Mais (Polenta) und Reis erhalten. Aus der letzten Tatsache glaubt Raubitschek auch für Beriberi den photodynamischen Ursprung annehmen zu müssen. Ich konnte aber niemals an weissen,

mit Reis gefütterten Tieren (Tauben, Ratten) irgendwelche Lichtwirkung beobachten.

Horbaczewski (190) kam wesentlich zu denselben Ergebnissen, wie auch Lode (191). Es scheint mir interessant, einige Punkte aus Horbaczewski's Arbeit besonders hervorzuheben. Bei der Fütterung von weissen Mäusen mit Maisgries, der mit Milch zusammengekocht wurde, traten folgende Symptome auf: Nach etwa 6 Wochen beginnen die Tiere sich heftig zu kratzen, besonders an den Ohren, die blutig infiltriert sind und oft allmählich abfallen; dieselben Erscheinungen treten auch am Schwanz auf. Dabei fallen die Haare stark aus, und schliesslich gehen alle Tiere an Gastroenteritis mit Blutungen zugrunde. Horbaczewski bemerkte nebenbei, dass die Lokomotion der Tiere sich abnorm gestaltete. Graue Mäuse dagegen zeigen bis auf einen merkwürdigen Wechsel der Haarfarbe keine Erscheinungen. Horbaczewski stellte aus dem Mais das Maisöl und den Maisfarbstoff dar. Die beiden Präparate wurden subkutan appliziert oder in die Haut eingerieben. Diese Injektionen waren von dem Ausbruch gewisser Krankheitssymptome gefolgt. Es bildeten sich an der Injektionsstelle kleine Geschwülste, aus denen der Farbstoff scheinbar nur sehr langsam resorbiert wurde. Die Haare gingen den Tieren aus, sonst aber konnten keine Erscheinungen zur Beobachtung kommen, die irgendwelche Analogien mit der menschlichen Pellagra hätten. Wurde dagegen das Maisöl oder der Maisfarbstoff in den Magen eingeführt, so konnten keine abnormen Erscheinungen zur Beobachtung gelangen, ausgenommen bei weissen Tieren. Subkutan appliziert treten dagegen bei Tieren jeder Farbe toxische Erscheinungen auf. Auch sollen einem der Eiweisskörper des Mais, dem Zein, auch toxische Eigenschaften zukommen. Horbaczewski kam im Gegensatz zu Raubitschek zum Schluss, dass auch die mit Alkohol extrahierte Polenta (weisse Polenta) auf weisse Tiere im Licht schädlich wirkt.

Der von Horbaczewski isolierte Maisfarbstoff, der zwar noch nicht mit aller Sicherheit rein dargestellt worden ist, krystallisiert in langen Tafeln und Nadeln, ist von roter Farbe; verdünnte Lösungen sind von rötlich-gelber Farbe, mit grüner Fluoreszenz. Im Gegensatz zu Raubitschek und Horbaczewski nimmt Umnus (197) als Ursache der Pellagra zwei Ursachen an: Eine toxische Substanz aus Mais und die photodynamische Wirkung des Maisfarbstoffs, der das Hauterythem verursacht. Die Maiskrankheit der Tiere und Pellagra hält er für identisch.

Seit der Publikation der photodynamischen Theorie haben sich schon viele Stimmen dagegen geäussert. Bei meinen Tauben, die mit rohem oder gekochtem Mais im Lichte gehalten wurden, konnte ich niemals eine schädliche Wirkung beobachten. Mais scheint für Tauben eine vorzügliche Nahrung zu sein. Lavinder (193) und Rondoni (194) konnten überhaupt die Lichtwirkung nicht bestätigen. Babes (195) glaubt nicht, dass guter Mais Pellagra

erzeugen kann. Hausmann (196) hebt hervor, dass die Hautläsionen in der Zeit, wo die Sonne am intensivsten wirkt, in der Regel schwächer auftreten. Wellman und Sparks (197) berichten über Winterfälle von Pellagra, die ohne merkliche Belichtung entstanden sind, Nutter (198) berichtet über Fälle bei Negern. Daffert und Kornauth (199) versuchten durch Alkoholextraktion des Mais giftige Substanzen auszuschleiden doch die Extrakte erwiesen sich als nicht toxisch, Hirschfelder (200) bemühte sich vergeblich, fluoreszierende Stoffe im Serum der Pellagrakranken nachzuweisen.

Beim Menschen kennen wir eine Dermatose (Hydroa aestivale, Sommereruption), bei welcher ein Lichtsensibilisator, nämlich Hämatoporphyrin die Eruption auslöst. Doch stammt diese Substanz höchst wahrscheinlich nicht aus der Nahrung, sondern entsteht durch Umwandlung des Blutfarbstoffs. Immerhin können wir ohne weiteres aus den übereinstimmenden Versuchen von Raubitschek, Horbaczewski und Lode entnehmen, dass im Mais ein Lichtsensibilisator vorhanden ist, der ähnlich wie Hämatoporphyrin wirkt. Diese Wirkung scheint überhaupt vielen fluoreszierenden Substanzen aus der Gruppe des Hämoglobins und Chlorophylls zuzukommen, die in unserer Nahrung immer vorhanden sind. Trotzdem kommen diese Lichtwirkungen nie zum Vorschein, weil eben die Lichtsensibilisatoren nur bei subkutaner Applikation ihre volle Wirkung entfalten können. Es fehlt vorläufig jeder Beweis, dass die hier beschriebenen Symptome irgend etwas mit der Pellagra zu tun haben. Die Tatsachen an und für sich sind aber sehr interessant. Alles was wir oben angeführt haben zeigt, dass der Alkohol-extrakt der Nahrung keine Giftstoffe enthält, sondern im Gegenteil Substanzen, die für das Leben unentbehrlich sind. Interessant erscheint mir hier anzuführen, dass Schöffner und Kuenen (201) ein Hauterythem, sehr ähnlich dem Pellagraerythem, bei 23% der Beriberipatienten in Ostindien beobachtet haben. Sollte man nicht diese Erscheinungen als Folge der Rückenmarkläsionen auffassen?

Die partielle Unterernährungstheorie.

Die kurze Zusammenstellung der für Pellagra heute geltenden Theorien zeigt uns zu Genüge, dass sich die Frage in demselben Stadium befindet, wie die Beriberifrage vor etwa 10 Jahren. Aus der sehr nahen Analogie der beiden Krankheiten, die ohne weiteres aus dem hier Gesagten hervorgeht, schien mir es sogar notwendig, schon früher auf den engen Zusammenhang beider Krankheiten hinzuweisen. Meine dahin zielenden Bemerkungen (l. c. 19) blieben jedoch bis jetzt unbeachtet. Da es bis jetzt nicht gelungen ist, bei Tieren durch Fütterung mit Mais (über diesbezügliche Versuche werden wir weiter unten berichten), einen Zustand hervorzurufen, den wir mit voller Berechtigung als experimentelle Pellagra bezeichnen könnten, müssen wir uns

vorläufig an die klinische Erfahrung halten. Das Problem das hier untersucht werden muss, lautet: Welches ist die exakte Zusammensetzung der Nahrung in den Pellagragenden? Liesse es sich hier zeigen, dass Mais nicht unbedingt daran schuld sei, so wäre dies auch kein Grund die Unterernährungstheorie abzulehnen, solange die Nahrung uns in mancher Beziehung ungenügend erscheint. Es ist wohl jetzt allgemein angenommen, dass die Ursache der Pellagra in der Maisnahrung zu suchen ist. Ist das der Fall, so muss untersucht werden, ob ein geringer Zusatz von Nahrungstoffen, die bekanntermassen Vitamine enthalten (Fleisch, Hefe, Milch und a. m.) ohne die Hauptdiät zu ändern, die Krankheit zum Stillstand bringen kann¹⁾. Wäre dies der Fall, so müssten alle früher angeführten Theorien entschieden abgelehnt werden. Leider liessen sich in der Literatur keine Angaben finden, die die oben skizzierten Fragestellungen näher behandeln.

Lombroso (l. c. 157) und Camurri (l. c. 162) beschreiben wohl eine Nahrung aus italienischen Pellagradistrikten, da aber dies wohl zu differenten Zwecken geschah, so kommen die Zahlen für unsere Zwecke kaum in Betracht.

Lombroso		Camurri	
Mais	1091 g täglich	Polenta	1500 g
Bohnen	60 „ „	Milch	100 „
Reis und Gerste	67 „ „	Reis	100 „
Kartoffeln	67 „ „	Kartoffeln	100 „
Gemüse	250 „ „	Gemüse	100 „
Speck	21 „ „	Speck	20 „
Olivöl	33 „ „	Olivöl	10 „
Fisch	67 „ „	Bohnen	100 „
Geflügel	27 „ „	Käse	50 „

Grosse Änderungen der Diät wurden in den verschiedenen Jahreszeiten beobachtet, die vielleicht das periodische Auftreten der Krankheit (im Frühling) erklären könnten. Es scheint, dass die italienische Landbevölkerung sich im Winter viel schlechter ernährt. Lombroso berichtet über solche Diätänderungen aus der Provinz der Ferrara.

Diät in den 8 Wintermonaten		Diät in 4 Sommermonaten	
Polenta	1000 g		160 g
Milch	—		—
Eier	fast gar keine		fast gar keine
Zwiebel	eine täglich		2 täglich
Malzbrot	50 g		400 g
Malzbrot	50 „		200 „

¹⁾ Lombroso berichtet über Fälle (l. c. 157), die durch Fleischzulage gebessert oder sogar geheilt wurden.

	Diät in den 8 Wintermonaten	Diät in 4 Sommermonaten
Fleisch	10 „	60 „
Käse	5 „	20 „
Bohnen	150 „	40 „
Fisch	20 „	wenig

Diese Nahrung scheint sehr einseitig zu sein und besteht zum grossen Teil aus Stärke, die ja bekanntlich als einzige Nahrung verabreicht Beriber erzeugt. Die übrigen Zusätze sind zu gering, um eine genügende Zufuhr von Vitaminen zu gestatten. Doch sehen wir, dass im Sommer entschieden eine bessere Nahrung Verwendung findet. Es erscheint mir aussichtsreich, der Bevölkerung zur Maisnahrung eine Kartoffelzulage zu geben.

Die Fütterungsversuche mit Mais bei Tieren gaben folgende Resultate.

Bezzola (202) zeigte, dass Mais keine ausreichende Nahrung darstellt, um Meerschweinchen in gutem Gesundheitszustand zu erhalten, sie verlieren Haare, bekommen starke Diarrhöe und gehen ein. Ein Unterschied bei der Verwendung von gutem und schlechtem Mais wurde nicht beobachtet. Lucksch (203) fand, dass Meerschweinchen, mit gutem Mais genährt, die Haare verlieren, eine Hyperämie der Darmmukosa und eine Vergrösserung der Nebennieren zeigen. Mit einem Gemisch von Mais, Mehl und Grünkraut genährt verlieren sie die Haare und erkranken an einer Lähmung der hinteren Extremitäten und Dünndarmkatarrh. Die Maisnahrung erwies sich ebenfalls als ungenügend für Kaninchen und Hunde, wobei im Frühling die erhaltenen Resultate deutlicher waren als im Herbst. Das Blut der Versuchstiere wurde ebenfalls untersucht und immer steril befunden. v. Neusser (l. c. 163) berichtet über eine Krankheit bei Pferden, die unter dem Namen Emmaisadura in Mexiko und Kolumbia bekannt ist, und die durch verdorbenen Mais verursacht sein soll. Als Symptome dieses Übels werden Abmagerung, Schwindel, Traurigkeit, Krämpfe, Tollheit, Ausfallen der Haare und der Zähne und Abfallen der Hufe angegeben. Holst (204) macht auf das häufige Auftreten skorbutischer Symptome bei Pellagra aufmerksam, speziell der Knochenporosität, die charakteristisch für den Skorbut ist. Er wiederholte Luckschs Experimente an Meerschweinchen und kam zu dem Ergebnis, dass er der von diesem Autor beschriebenen Krankheit, die Holst selbst als Skorbut bezeichnet, durch Zusatz von frischem Kohl vorbeugen konnte. Sogar der konstant auftretende Haarausfall konnte vermieden werden, obwohl die Experimente im Frühling zur Ausführung gelangten. Wir sehen aus dieser Zusammenstellung klar, dass die durch die Maisfütterung bei den Tieren hervorgerufenen Symptome kaum etwas mit der menschlichen Pellagra zu tun haben. Es erscheint jedoch nicht ausgeschlossen, dass weitere Forschungen ein Versuchstier ausfindig machen werden, das für Pellagra empfänglich ist. Zurzeit haben wir keine experimentellen Beweise, für die Ursache

der Pellagra in der partiellen Unterernährung anzunehmen. Doch entspricht die letztere Theorie meiner Meinung nach am besten den vorliegenden Tatsachen. Ausserdem muss auf manche Analogien der Pellagra mit dem Skorbut hingewiesen werden.

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Rachitis.

Trotz der ausserordentlichen Verbreitung dieser Volkskrankheit und der grossen Anzahl von wissenschaftlichen Arbeiten, die sich mit der Pathogenese dieser Krankheit befassen, ist die Ursache der Rachitis noch völlig dunkel. Es ist nicht meine Aufgabe, auf die Einzelheiten einzugehen und ich verweise hier auf die modernen Zusammenstellungen: (Zybell (205), Ludwig F. Meyer (206), Orgler (207), Wieland (208), Lehnerdt (209), und andere mehr, die das Thema bereits erschöpfend behandelt haben.)

Es ist wohl jetzt von der Mehrzahl der Kliniker angenommen, dass die Rachitis eine Stoffwechselkrankheit ist, die besonders in Grossstädten vorkommt und nach v. Hansemann eine Folge der Domestikation ist (210). Allerdings wird mehr und mehr von Fällen berichtet, die im hohen Norden und in den Tropen zur Beobachtung kamen. Es mangelt aber nicht an Stimmen, die sich der Meinung v. Hansemanns anschliessen und als Ursache der Krankheit schlechte sanitäre Bedingungen und die verdorbene Luft der Grossstädte ansehen. So führt z. B. Peiper (211) das Nichtvorkommen der Rachitis in Deutsch-Ost Afrika auf diese Momente zurück, er lässt aber vollständig ausser acht, dass die Eingeborenen in Afrika die künstliche Ernährung der Kinder wohl gar nicht kennen. Die statistischen Angaben über die Verbreitung der Krankheit (z. B. die Angabe, dass 90 % der Grossstadtkinder an Rachitis erkranken soll) leiden sehr an dem Übel, dass die Kliniker den Begriff der Rachitis noch nicht begrenzt haben. Deswegen ist die Frage der kongenitalen Rachitis und ebenso die Frage, ob typische Rachitis bei Brusternährten Kinder oft vorkommt, noch nicht gelöst, eine Frage, die für unsere Ausführungen von grossem Interesse ist und die weiter unten diskutiert wird. Das wesentliche Merkmal der Rachitis ist das Verharren des Knorpel- und Knochengewebes im unverkalkten Zustand. Das rachitische Knochengewebe wird hauptsächlich durch seinen abnorm geringen Gehalt an Erdalkali gekennzeichnet. Bei Rachitis ist die Ablagerung der Kalksalze in denjenigen Skeletteilen gehindert, die während des normalen Wachstums solche aufnehmen, und zwar im gesamten Skelett (Schmorl (212)).

Bei der Untersuchung der rachitischen Knochen findet man ein Überwiegen des Knorpels über das Knochengewebe, so findet Dibbelt (213) in normalem Knochen 29,43 % Knorpel, im rachitischen dagegen 71,29 %. Ausserdem findet man eine übermässige Bildung des osteoiden Gewebes wie Schmorl (212) und Veränderungen im Knochenmark, wie sie Marfan, Bardouin und Feuillé (214) und Hutinel und Tixier (215), beschrieben haben. Kassowitz (216) findet an Stellen, die in starkem Wachstum begriffen sind, wie zum Beispiel an der Epiphysengrenze, verstärkte Blutfülle, die eine Proliferation der Knorpelzellen verursacht und die Verkalkung derselben stören soll. Diese Ansicht fand viele Anhänger, aber auch viele Gegner. Heubner (217) und Schmorl und auch Pommer (218) finden in diesen Knochen keine Entzündung, sondern nur eine scheinbar abnorme Proliferation des Knorpelgewebes. Auch chemische Unterschiede wurden an rachitischen Knochen gefunden.

Dibbelt (l. c. 213) gibt folgende Zahlen an:

Normaler Knochen	Rachitischer Knochen
Knorpel 29,43	71,29
Fett 1,89	7,50
$\text{Ca}_3(\text{PO}_4)_2$ 57,38	15,11

	Normaler Knochen	Rachitischer Knochen
Phosphors. Magnesia	1,72	0,78
CaCO ₃	8,95	3,15
Salze	0,83	2,20

Gassmann (219) findet folgende Zahlen:

	Normal	Rachitis
CaO	24%	21%
P ₂ O ₅	33	30
CO ₂	3	2,75
MgO	0,10	0,53—0,74

In den rachitischen Knochen wurde ausserdem etwas mehr als 5% organische Substanz gefunden.

Ausser in den Knochen wurden von Hagenbach und Burekard (220) und von Bing (221) in schweren Fällen Muskeldystrophien aufgefunden¹. Als auf einer ähnlichen Stoffwechselanomalie beruhend möchten wir auch die Spasmophilie und Tetanie betrachten und wahrscheinlich auch die Osteomalazie. Wir wollen nun zu der Besprechung der Theorien kommen, die für die Entwicklung der Rachitisfrage eine nicht untergeordnete Rolle spielten. Gleichzeitig wollen wir die dazu gehörenden experimentellen Arbeiten diskutieren.

Domestikationstheorie.

Hansemann, Kassowitz (223) suchen die Ursache der Rachitis in den ungünstigen sanitären Verhältnissen, dunkeln und engen Wohnungen der ärmeren Bevölkerung der Grossstädte, Mangel an frischer Luft, etc. Kassowitz findet Rachitis viel seltener bei der Landbevölkerung, ferner häufiger am Ende des Winters, als am Ende des Sommers. Im Einklang mit dieser Anschauung fanden Haushalter und Sabatier (224), dass Hühner, die im Dunkeln und in wenig erneuerter Luft gehalten wurden Knochenveränderungen zeigten, die eine gewisse Ähnlichkeit mit den rachitischen haben sollen. Es muss aber berücksichtigt werden, dass bei der Landbevölkerung die Kinder nur selten künstlich ernährt werden, ferner dass die ungünstigen Verhältnisse der ärmeren Grossstadtteile auch auf die Mütter und folglich auf den Wert der Brustmilch, sowie auf die Qualität der künstlichen Kindernahrung ungünstig wirken.

Theorie, die den Mangel an Kalk oder eine gestörte Assimilation desselben als die Krankheitsursache ansehen

Eine ganze Reihe von Forschern kam auf den Gedanken, dass die Milch und speziell Muttermilch eine Kalkmenge enthält, die unter besonderen Be-

¹) Aschenheim und Kaumheimer (222) fanden in schweren Rachitisfällen den Gehalt der Muskeln an Kalk ebenfalls vermindert.

ungen, wie bei intensivem Wachstum, nicht ausreicht. Als Vertreter dieser Ansicht muss man vor allem Aron (225) nennen, der mit einer Reihe von Stoffwechselversuchen mit Kalk den Nachweis zu führen konnte, dass die Frauenmilch eine Kalkmenge enthält, die gerade an der Grenze des Bedarfs des Säuglings liegt. Die von Aron angewandte Methode scheint jedoch nicht ganz einwandfrei zu sein. Er nimmt als Basis für die Berechnungen die chemische Zusammensetzung des Neugeborenen, undchnet aus dem Gewichtszusatz die nötige Kalkmenge aus. Orgler (l. c. 207), hebt in seiner ausführlichen Besprechung des Kalkstoffwechsels bei Rachitis, wichtigerweise Bedenken gegen die von Aron benutzte Methode, indem bemerkt, dass das Wachstum nicht unbedingt mit dem Gewichtszuwachs übergeht. Ein zweiter Weg besteht darin, dass der Kalkbedarf bei rachitischen Kindern festgestellt, und daraus der normale Kalkbedarf berechnet wird. Auch dieser Weg ist nicht einwandfrei, da Kinder, die klinisch noch keine Rachitis zeigen, schon eine Stoffwechselstörung haben können, die später zu der Entwicklung dieser Krankheit führen kann. Andererseits können Säuglinge, die klinisch das Rachitisbild aufweisen, sich in voller Genesung befinden und normalen Stoffwechsel zeigen. Cronheim und Erich Müller (226) hatten offenbar mit solchen Fällen zu tun, die in Bezug auf den Kalkstoffwechsel sich normal verhielten. Sie untersuchten die Ausnutzung der rohen, gekochten und sterilisierten Milch und fanden eher eine bessere Ausnutzung der gekochten Milch. Ich vermute, dass die von diesen Autoren gewählten Versuchsperioden (4 Tagen) zu dem Schluss nicht berechtigt erscheinen lassen.

Während die Annahme einer zu geringen Kalkmenge in der Nahrung bestätigt wurde, scheint eine geringere Ausnutzung des Kalkes bei Rachitikern eine Tatsache zu sein, die durch zahlreiche Untersuchungen von Schabad (228) eine feste Stütze bekam. Er untersuchte in dieser Beziehung viele Kinder und die daraus gezogenen Schlüsse scheinen wohlbegründet zu sein. Die Anhänger dieser Theorie, zu denen auch Dibbelt (l. c. 213, 228) zu rechnen ist, untersuchten den Einfluss der Diät auf die Ausnutzung des Kalkes und kamen zu sehr interessanten Ergebnissen. Während man den Einfluss des Nahrungs eiweisses, welcher von L. F. Meyer (229) untersucht wurde, nur soviel bekannt ist, dass die Stickstoffausscheidung nicht parallel mit der Kalkausscheidung geht, ist über den Einfluss der Kohlehydrate und Fette viel mehr bekannt. Die Versuche von L. F. Meyer (l. c. 229), Rothberg (231) und Orgler (232) haben gezeigt, dass durch Zusatz von Fett, etwa in Form von Vollmilch, die Kalkausnutzung viel schlechter wird, und zwar infolge von Kalkverlusten im Kot in Form von Fettseifen. Doch ist dieser Einfluss des Fettes nicht etwa konstant, es finden sich Kinder, die diesen Einfluss vermissen lassen. Man geht aus den Experimenten von Dibbelt (233), Massanek (234), Tade

(l. c.) und Rothberg (l. c.), dass der Einfluss der Kohlehydrate nicht immer in demselben Sinne verläuft. Doch scheint der Einfluss eher günstig zu sein. Orgler glaubt nicht, dass die den Kalkstoffwechsel schädigende Wirkung des MilCHFettes allein durch die Verluste an Fettseifen zu erklären ist und meint, dass der Grund dafür im Kinde selbst zu suchen sei.

Sehr interessant ist die günstige Wirkung des Phosphorlebertran (Kassowitz) auf den Kalkstoffwechsel bei Rachitikern, die u. a. von Schabad (235) und Birk (236) bestätigt wurde. Wie Schabad fand, besitzt Phosphorlebertran allein, in gleicher Menge zugesetzt, nicht die günstige Wirkung. Lebertran allein wirkt nahezu ebenso günstig, wie Phosphorlebertran. Dagegen hat Lebertran nach Untersuchungen von Birk keine Wirkung auf gesunde Kinder. Die antagonistische Wirkungsweise von MilCHFett und Lebertran scheint mir das interessanteste Ergebnis zu sein, welches die obige Theorie zutage fördert und scheint einer weiteren Untersuchung wert zu sein. Eine Erklärung für die Tatsache, warum der Rachitiker den Kalk der Nahrung schlecht ausnutzt, blieb von den Anhängern dieser Theorie unbeantwortet. Es ist nicht ohne Recht betont worden, wie das schon von Pommer vor langer Zeit geschah, dass der Grund der Kalkstoffwechselstörung in einer allgemeinen Ursache zu suchen sei. Als solche hat Pommer eine Störung im Zentralnervensystem angenommen. Mit aller Vorsicht scheint sich auch neuerdings Schabad (238) dieser Ansicht anzuschließen. Auch L. Mohr (238) findet in der Mehrzahl der Rachitisfälle Erkrankung der Muskeln und Nerven. Die Suche nach einer allgemeinen Ursache der Rachitis führte viele Forscher dazu einen Zusammenhang mit den Drüsen ohne Ausführungsgang zu suchen. Es entstand auf diese Weise eine ganze Anzahl von Arbeiten, die jetzt besprochen werden.

Rachitis und Drüsen ohne Ausführungsgang.

Stoeltzner (238) bezeichnet die Nebenniere als das Organ, dessen Erkrankung Rachitis zur Folge haben soll. Er stützt sich dabei auf die anatomische Kleinheit derselben, geringen Gehalt an Adrenalin, die geringe Ausbildung des chromaffinen Gewebes bei rachitischen Kindern und die guten Erfolge der Nebennieren- und Adrenalintherapie. Im Gegensatz zu Stoeltzner konnten Schmorl und Cattaneo (239) bei der Rachitis keine Veränderung in den Nebennieren nachweisen. Die Angabe von Stoeltzner, dass durch Exstirpation einer Nebenniere bei Hunden rachitische Knochenveränderung erzeugt wurden, konnte von Jovane und Pace (240) nicht bestätigt werden.

Ebensowenig liess sich ein Einfluss der Thymus auf die Rachitis feststellen. Diese Ansicht, die von v. Mettenheimer (241) zuerst ausgesprochen wurde, ist von Mendel (242) neuerdings wieder aufgenommen worden. Er stützt sich dabei auf die Untersuchungen von Friedleben (243), der einen Zusammenhang zwischen der Drüse und dem Knochenwachstum annahm und

physiologische Bedeutung gesunder Kinder, insbesondere Nahrungsbestandteile, S. 114. 191.

Rachitisfällen ein Zurückbleiben in der Entwicklung der Thymus feststellte. Im Gegensatz dazu fand Du Castel (244) bei Rachitis die Thymus hypertrophiert. Die Arbeiten von Basch (245), Neumann (246), Klose und Vogt (247) verneinen jeden Zusammenhang zwischen Thymus und Rachitis.

Für die Schilddrüse haben Heubner (248), Knöpfelmacher (249) und Lanz (250) schon vor einer ganzen Reihe von Jahren gezeigt, dass die Beziehung mit der Rachitis in keiner Beziehung steht. Hönnike allerdings (251) nimmt eine Beziehung an. Nach Exstirpation der Epithelkörper fand Erdheim (252) bei Ratten-Tetanie fehlende Verkalkung des Dentins, sowie unvollständige Knochenverkalkung, angeblich analog dem Knochenbefund bei Rachitis. Doch verhalten sich die meisten Rachitisforscher (u. a. Schmorl (253)) ablehnend gegen die s. g. experimentelle Rachitis der Tiere.

In jüngster Zeit sind einige Arbeiten erschienen, die sich mit der Anwendung von Hypophysenpräparaten beschäftigen. Auf die Versuche von Babcock (253) und Neu (254) gestützt, die imstande waren durch Hypophyse bzw. Pituitrindarreichung einige Osteomalaziefälle günstig zu beeinflussen, versuchte Klotz (255) diese Therapie auch bei Rachitis. Er berichtet über sehr günstige Resultate, die sich besonders in einer allgemeinen Stimulation des Stoffwechsels äusserten. Wir müssen hier weitere Angaben abwarten, bevor wir uns über den Wert dieser neuen Therapie aussprechen können. Wenn wir die Ergebnisse dieser hier mitgeteilten Arbeiten kurz zusammenfassen, kommen wir zu der Ansicht, dass die Gründe, aus denen das rachitische Kind den Kalk aus der Nahrung schlechter als das normale, assimiliert, noch völlig unbekannt sind. Deshalb möchte ich zur Erklärung der Rachitis eine neue Theorie vorschlagen, die partielle Unterernährungstheorie, die aus den bisherigen Kenntnissen der Zusammensetzung der Kindernahrung sich glatt ableiten lässt¹⁾.

Die partielle Unterernährungstheorie der Rachitis.

Diese Theorie vermutet, dass Rachitis als Folge einer vitaminarmen Nahrung anzusehen ist. Bei Aufstellung dieser Theorie stütze ich mich auf die von mir ausgeführte Isolierung des Beriberivitamins aus der Milch. Weitere Versuche ergaben (noch nicht veröffentlicht), dass der Vitamingehalt der Milch grossen Schwankungen unterworfen ist. Einige Beweise können schon jetzt angeführt werden. Als solche Beweise gelten wir 1. das seltenere und mildere Vorkommen der Rachitis bei brustnährten Kindern und 2. die altbewährte günstige Wirkung der Brustnahrung

¹⁾ Ausser den genannten Theorien gibt es noch andere, wie die von Esser (256), der die überreichen Ernährung die Ursache der Rachitis sucht, sowie die von Ribbert (257), der eine toxische Wirkung falsch zusammengesetzter Nahrung annimmt.

bei rachitischen Kindern. Die ungenügende und oft unzweckmässige Nahrung der stillenden Mütter, die schlechtere Nahrung der Kühe im Winter, der Einfluss des langen Kochens auf den Vitamingehalt der Kuhmilch, die Ernährung der Kinder mit vitaminarmen Mehlen, der Einfluss eines Nahrungswechsels auf rachitiskranke Kinder, die Wirkung des Lebertrans, dies sind alles Punkte, die für unsere Auffassung sprechen. Was die angeborene Rachitis betrifft [ihre Existenz wird übrigens von manchen Pädiatern in Abrede gestellt, Wieland (258) so wird sie auch durch unsere Theorie leicht erklärbar. Wir wollen nur auf das Vorkommen von infantiler Beriberi bei beriberikranken Müttern (Andrews l. c. 36) erinnern. Es wäre möglich anzunehmen, dass kongenitale Rachitis nur dann vorkommt, wenn bei der Mutter schon eine Stoffwechselanomalie besteht, während bei brusternährten Kindern nur dann Rachitis ausbricht wenn die Milch der Substanz entbehrt, die den Kalkstoffwechsel reguliert. Es darf nicht vergessen werden, dass auch Beriberi noch vor kurzem als eine Anomalie des Phosphorstoffwechsels angesehen worden ist. Die meisten Rachitisfälle kommen jedoch etwa im 6. Monate vor, wir müssen daher zuerst die Nahrung des Säuglings näher ins Auge fassen. Da wir die Milchfrage im Kapitel über Barlowsche Krankheit schon ausführlich behandelt haben, brauchen wir auf dieselbe nicht mehr einzugehen. Dagegen kommen noch die Mehle in Betracht, die sich zeitweise grösserer oder geringerer Beliebtheit erfreuen.

In Folge der Einführung der Noordenschen Haferkuren bei Diabetikern wurden die verschiedenen Mehlsorten einer eingehenden Untersuchung unterworfen. Die sehr interessanten Befunde von Max Klotz (259) haben gezeigt, dass zwischen den einzelnen Mehlen weitgehende biologische Unterschiede bestehen. Er fand (260), dass erstens beim diastatischen Abbau Hafermehle mehr Maltose und weniger Glukose ergeben, als Weizenmehle. Hafer soll auch durch Fermente und Bakterien leichter abgebaut werden als Weizen. Die Tatsache, dass Hafermehl bei Diabetikern oft die Zuckerausscheidung im Harn nicht erhöht, wird auf die Weise erklärt, dass Hafermehl zum Unterschied von Weizenmehl von der Darmbakterienflora so energisch abgebaut wird, dass nur Abbauprodukte der Glukose auf anhepatischem Wege zur Resorption gelangen. Diese Ansicht wird von Klotz vertreten¹⁾. Er glaubt, dass bei fleischfreier Diät eine kräftige amylytische Bakterienflora sich entwickelt. Dagegen wird für Kindermalzsuppen das schwerer abbaubare Gerstenmehl allgemein empfohlen. Auch bei der Tetanie der Kinder wird vor Fischbein (262) und Escherich (263) Mehlnahrung empfohlen. Wir haben in den früheren Kapiteln gesehen, dass einige Mehle im allgemeinen arm an Vitaminen sind. Während die Antiskorbuts substanz darin vollständig zu

¹⁾ Wieland (261) konnte die bessere Ausnützung des Hafers beim Diabetiker nicht feststellen. Er glaubt, dass es sich hier, gegenüber anderen Mehlsorten nur um graduelle Unterschiede handelt.

nen scheint, verhalten sie sich in bezug auf das Erzeugen von Beriberi verschieden. Wir sahen, dass z. B. das Gerstenmehl das Beriberivitamin enthält. Es wäre möglich, dass die verschiedene Ausnützung der Mehle (Hafermehl bei Diabetikern) nicht etwa auf die verschiedene Abbauweise der Mehle, sondern auf die Gegenwart einer Substanz von Vitamintypus zurückzuführen ist, die eine Ausnützung der Glukose beim Diabetiker gestattet. Dieser Gedanke ist mir von Prof. MacLeod ausgesprochen worden. Was die Darreichung der Mehle bei Säuglingen anbetrifft, so muss zu grosser Vorsicht mahnt werden. Die Mehlnahrung, infolge ihrer Armut an Vitaminen, darf, meiner Ansicht nach, nur einen geringen Teil der gesamten Nahrung im Säuglingsalter ausmachen. Die Einführung dieser neuen Theorie gestattet neue Versuchsreihen anzustellen, die mir dringend notwendig erscheinen. Vor allem muss untersucht werden, ob alkoholische Extrakte aus leicht erhitzter Milch den Zustand der Rachitis zu bessern vermögen. Auch die günstige Wirkung des Lebertrans bei Rachitis macht es nicht unwahrscheinlich, dass auch der Lebertran die Substanz enthält, die den Kalkstoffwechsel bei rachitischen Kindern zu bessern vermag. Es wäre vielleicht möglich, durch ein passendes Konzentrationsverfahren, die Wirkung des Lebertrans noch günstiger zu gestalten. Auch halte ich für ratsam, den stillenden Frauen frisches rohes Obst, resp. Frucht- und Gemüsesaft von rohen Pflanzen zu verabreichen. Fruchtsaft aus rohem Obst wird hoffentlich auch bei rachitischen Kindern bewähren. Ich habe die Absicht, die Untersuchung der Rachitis vom Standpunkt der partiellen Unterernährung aufzunehmen.

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Die Vitamine in ihren Beziehungen zu der Ernährungs- und Stoffwechsellehre. Schlussbetrachtungen.

Mit der Auffindung der Vitamine in unserer Nahrung eröffneten sich mit einem Schlage neue Gesichtspunkte, die unsere bisherigen Anschauungen über die Ernährung und Stoffwechsel sehr stark beeinflussen werden. Jetzt

wissen wir sicher, dass die Bewertung der Nahrung auf Grund ihres Gehalts an Proteinen, Kohlehydraten und Fetten, sowie ihres Kalorienwertes nicht mehr ausreicht. Sahen wir doch, dass eine in dieser Beziehung tadellose Nahrung sich als vollständig unzureichend erweisen kann, wenn ihr die Vitamine fehlen.

Vitamine und Ernährung.

Im letzten Jahrzehnt stand die Ernährungslehre im Zeichen des Eiweissstoffwechsels. Durch die Arbeiten Emil Fischers angeregt, suchten viele Forscher den Wert verschiedener Eiweisskörper für die Nahrung festzustellen. Als Resultat dieser Forschungen konnte gezeigt werden, dass den einzelnen Eiweisskörpern ein verschiedener Nahrungswert zukommt, je nach Gehalt und Zusammensetzung an Aminosäuren. Eiweisskörper, denen gewisse Aminosäuren fehlen, erwiesen sich als nicht ausreichend. Zu den lebenswichtigen Aminosäuren können auf Grund vieler Untersuchungen Tyrosin, Phenylalanin und Tryptophan mit aller Sicherheit gerechnet werden. Fehlen dem Organismus diese Substanzen, so ist erstens ein kompletter Aufbau des arteigenen Eiweisses, zweitens eine Umarbeitung dieser Aminosäuren in gewisse lebenswichtige Substanzen unmöglich gemacht. Genau so wie die Pflanze gewisse aus den Aminosäuren entstehende Ringkomplexe höchstwahrscheinlich für den Aufbau der Alkaloide benutzt, wäre es denkbar, dass der tierische Organismus dieselben zu ähnlichen Zwecken braucht. Es wurde schon öfters die Ansicht ausgesprochen, dass Tryptophan die Muttersubstanz des Blutfarbstoffs, Tyrosin und Phenylalanin (allerdings auf Umwegen, vielleicht über das Homogentisinsäurederivat des Tyrosins) dagegen die des Adrenalins sei.

Ich stelle mir vor, dass die physiologische Wichtigkeit der Vitamine auf einem ähnlichen Prinzip beruht und habe übrigens diese Ansicht schon früher (l. c. 22) ausgesprochen. Ihre Wirksamkeit in minimalen Mengen macht es höchst wahrscheinlich, dass sie in einer nahen Beziehung zu den Hormonen und den Sekreten der Drüsen ohne Ausführungsgang stehen. Diese Produkte wurden allerdings von diesem Standpunkt aus noch nie untersucht. Da die Vitamine eine Gruppe von scheinbar sehr wenig reaktionsfähigen Körpern gehören, die keine charakteristischen Derivate bilden, so wäre es interessant, diese Produkte chemisch mit derselben Methodik, wie dies bei den Vitaminen geschah, zu untersuchen. Vor allen Dingen müsste festgestellt werden, ob die Drüsen selbst prozentual einen grösseren Vitamingehalt besitzen als die übrigen Gewebe. Ist das der Fall, so muss die Silbernitratfraktion einer sorgfältigen Untersuchung unterworfen werden. Auch auf diese Frage hoffe ich bald näher einzugehen. Als erster Schritt in dieser Richtung kann die Arbeit von Dale und Laidlaw (264) bezeichnet werden, die eine Reinigung von Sekretin mit einer ähnlichen Methode vornahmen.

Wenn auch eine grosse Analogie zwischen gewissen Aminosäuren und Vitaminen besteht, es sind doch wichtige Unterschiede vorhanden. Die Frage wird nämlich dadurch komplizierter, dass die Vitamine zum Unterschied von den Aminosäuren sehr instabil sind. Und zwar finden wir darin weitgehende Unterschiede, das Skorbutvitamin ist viel labiler, als das Beriberivitamin. Nicht nur das Erhitzen, sondern auch das Trocknen ist für das Skorbutvitamin schädlich. Diese letzte Frage kommt auch für die Landwirtschaft in Betracht. Fleischmann (265) konnte zeigen, dass Kälber, die mit Heu gefüttert werden, sehr oft erkranken. Es scheint demnach, dass beim Trocknen des Heues Stoffe verloren gehen, die unentbehrlich sind. Tatsächlich konnte er zeigen, dass das Lecithin darin durch Bakterienwirkung zerstört wird, Phosphatide und Eiweiss dagegen durch Zelltätigkeit zerstört werden und zwar die ersten bis 87%, Eiweiss 10—15%. Der Gehalt an Gesamtstickstoff bleibt aber unverändert. Von Pächtner (266) wurde gefunden, dass aufgekochte Frischhefe die Milchproduktion bei Kühen erheblich bessert. Die vorliegende Frage ist von grossem Interesse, da die Kuhnahrung im Winter mangelhaft ist, wodurch eine vitaminarme Milch erzeugt wird.

Die Erforschung der Vitamine hat ferner eine grosse Bedeutung für unsere Kenntnisse der synthetischen Fähigkeiten des tierischen Körpers. Hier können wir weitere Substanzen kennen lernen, die der tierische Organismus offenbar nicht zu synthetisieren vermag, sondern fertig aus dem Pflanzenreich beziehen muss. Es muss dies um so mehr betont werden, da in letzter Zeit durch die neueren Arbeiten von Embden (267), Grafe (268) und Abderhalden (269) der Anschein erweckt werden konnte, dass durch Zufuhr von Ammoniaksalzen bei grossen Kohlehydratgaben das Eiweiss ersetzt werden, oder jedenfalls der Stickstoffbedarf zum grossen Teil gedeckt werden könnte. Durch neue Arbeiten von Grafe (270) und Abderhalden (271) konnte jedoch gezeigt werden, dass die Zufuhr von Ammoniaksalzen zwar eiweiss-sparend wirkt, aber nicht vollständig das letztere ersetzen kann. Es handelt sich wahrscheinlich nur, wie dies auch von den obengenannten Autoren richtig interpretiert wurde, um Ersparnis der Aminosäuren, die sonst einer Desaminierung anheimfallen würden. Die synthetische Fähigkeit des tierischen Organismus konnte bei den Aminosäuren nur für die niederen Glieder der Reihe, nämlich für Glykokoll und Alanin (Durchblutungsversuch der Leber mit Brenztraubensäure und Ammoniaksalzen) einwandfrei bewiesen werden.

Seit meiner früheren Zusammenfassung (l. c. 22), worin ich auf die Bedeutung und Notwendigkeit der Berücksichtigung der Vitamine bei den Stoffwechselversuchen hinwies, sind einige Arbeiten erschienen, die diesen Einfluss einer experimentellen Prüfung unterzogen. Vor allen Dingen muss hier die Arbeit von Suzuki, Shimamura und Odake (l. c.) erwähnt werden. Sie fütterten Hunde mit poliertem Reis und mit Pferdefleisch, das mit Wasser extrahiert worden war. Nach etwa 4 Wochen zeigten die Tiere

unter allmählicher Gewichtsabnahme Appetitlosigkeit und allgemeine Schwäche. Wurde nun den Hunden alkoholischer Extrakt der Reiskleie in kleinen Dosen verabreicht, so erholten sich die Tiere zusehends, nahmen an Gewicht zu und frassen mit grosser Gier. Auf diese Weise wurde ein Hund 98 Tage am Leben erhalten, zwei andere lebten 223 resp. 120 Tage. Ein alkoholischer Extrakt aus Pferdefleisch soll dieselbe Wirkung besitzen, wie der aus Reiskleie. Auch bei anderen Tieren wurde von den obengenannten Autoren eine günstige Wirkung der Reiskleie festgestellt.

Andererseits hat Abderhalden (272), obwohl er scheinbar meinen Angaben noch skeptisch gegenübersteht, den Einfluss der Vitamine auf den Stoffwechsel studiert. Er konnte keine Wirkung konstatieren, was daher kommt, dass den Hunden erstens eine sonst unvollwertige Nahrung (Gelatine, Ammonsalze etc.) verabreicht wurde (Vitamine können natürlich nicht die fehlenden Aminosäuren ersetzen), zweitens wurde der Einfluss studiert, als noch die Versuchstiere selbst genug Vitamine aus der vorigen kompletten Nahrung stammend zur Verfügung hatten. Wir sahen in den vorigen Kapiteln, dass die verschiedenen Versuchstiere sehr verschiedene Quantitäten Vitamine aufspeichern. Tauben erkrankten etwa nach 2—4 Wochen, Meer-schweinchen nach 4 Wochen, Hühner nach 3—4 Wochen, Kaninchen nach etwa 3 Monaten, Hunde nach etwa 4—6 Wochen, Mäuse nach etwa 6—12 Tagen, während Ratten erst nach viel längerer Zeit zugrunde gehen.

Will man nun aus einem Stoffwechselversuch den Schluss ziehen, dass die Versuchsnahrung in jeder Beziehung ausreicht, so müssen die Versuchsperioden bedeutend länger gehalten werden, als oben angegeben, um auch die individuellen Schwankungen Rechnung zu tragen.

Hier wäre noch eine Arbeit von Grafe (273) zu erwähnen. Er fütterte zwei Schweine mit Stärke, Zucker und Ammoncitrat. Dem zweiten Tiere wurde ausserdem noch eine Fleischabkochung zugesetzt. Das zweite Tier zeigte noch nach 32 Tagen ein fast unverändertes Gewicht und auch eine bessere Ausnutzung der Ammonsalze.

Wir sind hier zum Schlusse unserer Ausführungen angelangt. Allerdings kamen hier nur die extremen Fälle zur Besprechung, wo das Fehlen der Vitamine Massenerkrankungen zur Folge hat. Ob ein momentanes Fehlen der Vitamine in der Nahrung, oder eine nur unvollständige Zufuhr derselben, nicht auch Störungen zur Folge haben kann, können wir noch nicht sagen. Es wäre jedoch denkbar, dass auch in diesem Falle vorübergehende Erkrankungen auftreten können. Es eröffnet sich hier ein neues Gebiet für klinische und physiologische Untersuchungen.

Die Diätlehre muss nun an der Hand der hier aufgestellten Prinzipien einer Revision und Reform unterzogen werden, und für die richtige Zusammensetzung und Zubereitung unserer Nahrung sind neue Fragestellungen entstanden, die erst durch Bestimmung des relativen Vitamingehalts der Nahrungsstoffe beantwortet werden können.

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Nachtrag zum Aufsatz: Über die physiologische Bedeutung gewisser bisher unbekannter Nahrungsbestandteile, der Vitamine.

Von

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Nachtrag zur Isolierung der Vitamine aus Reiskleie und Hefe.

Vor kurzem ist es mir gelungen die Vitaminfraktion aus den oben genannten Ausgangsmaterialien (Casimir Funk, Brit. Med. Journ. April 19, 1913, Journ. of Physiol. im Druck) in einige Substanzen zu zerlegen. Aus Hefe konnten drei Substanzen isoliert werden, nämlich eine Substanz von der Formel $C_{24}H_{19}O_9N_5$, eine andere von der Formel $C_{29}H_{23}O_9N_5$ und Nikotinsäure. Die Tierversuche haben ergeben, dass die Substanzen einzeln den Tauben verabreicht nur eine sehr geringe Wirkung besitzen; wurde aber die erste Substanz zusammen mit der Nikotinsäure in Mengen von einigen Milligrammen subkutan verabreicht, so wurde eine sehr frappante Heilwirkung erzielt.

Aus Reiskleie wurden auf dieselbe Weise zwei Körper isoliert, nämlich einer von der Formel $C_{26}H_{20}O_9N_4$ und Nikotinsäure. Über die Wirkung der beiden Substanzen wird später berichtet werden. Es ist sehr wahrscheinlich, dass alle oben beschriebenen Substanzen von einer Muttersubstanz abstammen, bei deren Aufbau die Nikotinsäure eine grosse Rolle zu spielen scheint.

Nachtrag zur Krebsfrage.

Das Roussche Sarcoma der Hühner wird von seinem Entdecker immer mehr als eine Infektionsgeschwulst betrachtet. Er konnte nämlich zeigen (Rous and Murphy, Rock. Inst. Coll. Pap. XVI, 1913), dass sehr dichte Filter (Chamberland F.) das Agens nicht durchlassen und dass das Agens durch Autolyse und durch Zusatz von Antiseptica sowie beim Erhitzen auf $50^{\circ}C$ zerstört wird. Meine noch unveröffentlichten Versuche haben dagegen gezeigt, dass wenn man das trockene Tumorgewebe 30 Stunden mit absolutem

Alkohol schüttelt, dann abfiltriert und den alkoholischen Extrakt im Vakuum einengt, den Rückstand in Wasser emulgiert und Hühnern einspritzt, grosse Tumoren mit Metastasen entstehen, die den Bau des ursprünglichen Tumors wohl bewahren und die sich weiter impfen lassen. Auch mit dem Filterrückstand liessen sich Tumoren, wenn auch nach längerer Inkubation, erzielen. Diese Befunde, wenn sie auch noch nicht ganz die Infektionsmöglichkeit ausschalten, legen den Gedanken nahe, dass das Agens, welches bei Hühnern das Roussche Sarcoma erzeugt, eine chemische Substanz vom Vitamintypus, darstellt. Weitere Versuche über diese interessante Frage sind im Gange.

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Fortschritte der experimentellen Beriberiforschung in den Jahren 1911—1913.

Von Dr. Casimir Funk (Beit Memorial Research Fellow).

Als ich in der Mitte des Jahres 1911 meine ersten Studien über Beriberi unternahm [1], war über die Natur der im polierten Reis fehlenden Substanzen folgendes mit Sicherheit festgestellt:

1. Die wirksamen Substanzen sind löslich in Wasser, Alkohol und säurehaltigem Alkohol;
2. sie sind dialysierbar;
3. sie werden durch Erhitzen auf 130° zerstört.

Wie wir daraus ersehen können, war über die Natur der fehlenden Substanzen sehr wenig bekannt. Auch über die Aetiologie der Krankheit selbst wurden und werden noch jetzt die allerverschiedensten Ansichten geäußert, die nicht gerade dazu helfen, Licht in die interessante und wichtige Beriberifrage zu werfen. Die hier folgende kurze Darstellung wird uns zeigen, wie sich die Frage in den letzten 2 Jahren entwickelt hat und welche Ideen sich daran unmittelbar anknüpfen lassen.

Zuerst musste entschieden werden, ob die hier in Frage kommenden wirksamen Substanzen chemischen Eingriffen widerstehen können und zu welcher Gruppe der chemischen Substanzen dieselben zu zählen wären. Meine ersten Versuche zeigten, dass die Wirkung der Hefe zum grossen Teil erhalten bleibt, wenn dieselbe 24 Stunden mit starker Schwefelsäure hydrolysiert wird. Diese grosse Widerstandsfähigkeit den starken Säuren gegenüber machte es von vornherein sehr wahrscheinlich, dass es sich in diesem Falle um eine stickstoffhaltige Substanz handelt. In dieser Weise wurde die weitere Richtung klar erkannt und systematischer Bearbeitung zugänglich gemacht. Es ist ganz klar, dass nur eine systema-

tische Untersuchung hier zum Ziele führen konnte. Eine ganze Anzahl von Untersuchern, deren Namen ich hier nicht weiter nennen will, hat das erste beste aus dem Gemisch krystallisierende Produkt als die wirksame Substanz angenommen. Wir müssen ja nicht vergessen, dass wir in solchen Extrakten mit grossen Gemischen zu tun haben, die unter Umständen aus Hunderten von bekannten und unbekannten Substanzen bestehen können.

Nachdem die stickstoffhaltige Natur der wirksamen Substanz durch meine Versuche sehr wahrscheinlich gemacht worden ist, ging ich an die Fraktionierung der Extrakte. Die von mir angewandte Methode war kurzgefasst folgende. Die zu untersuchenden Futterstoffe wurden mit Alkohol in der Kälte extrahiert, eine Methode, die gegenüber der früher benutzten von grossem Vorteil war, da die Extrakte von Verunreinigungen, alkoholunlöslichen Stoffen zum grossen Teil befreit wurden. Die alkoholischen Extrakte wurden daraufhin mit Methoden bearbeitet, die für die Fraktionierung von stickstoffhaltigen Substanzen üblich sind, mit einigen Modifikationen. Durch Anwendung von Tauben¹⁾, die mit poliertem Reis in den Zustand der experimentellen Beriberi versetzt worden sind, als eine Art von Indikator, wurde jede erhaltene Fraktion auf die Gegenwart der wirksamen Substanz geprüft. Es konnte nun festgestellt werden, dass die wirksame Substanz in den Niederschlag übergeht, der bei der Anwendung von Phosphorwolframsäure entsteht. Die Substanz wird ferner mit Sublimat in alkoholischer Lösung und mit Silbernitrat teilweise, mit Silbernitrat und Baryt dagegen nahezu quantitativ gefällt. Bei der Fraktionierung der Reiskleie [2] konnte auf diese Weise aus der letzten Fraktion eine neue stickstoffhaltige Substanz in geringen Mengen isoliert werden, der schon damals und mit Recht eine Bedeutung für die Heilwirkung zugesprochen worden ist. Später konnten mit der gleichen Methodik ähnliche Substanzen auch aus der Hefe [3], Milch [4],

¹⁾ Eijkman in einer vor kurzem erschienenen Arbeit (Archiv f. Schiffs- und Tropenhygiene, 17, 328, 1913) glaubt, dass Tatsachen, die aus dem Studium an Tauben gewonnen wurden, nicht ohne weiteres auf Hühner übertragen werden können. Er fand nämlich, dass NaCl und KCl, in gewisser Menge Tauben injiziert, dieselben heilen, während bei Hühnern keine Wirkung festgestellt wurde. Ich habe diese Angaben genau nach Eijkmans Vorschrift an 5 Tauben nachgeprüft und gefunden — wie das aus meinen Arbeiten zu erwarten war —, dass dieses Mittel ohne die geringste Wirkung auf kranke Tauben ist. Eijkmans Angabe scheint somit nicht zu stimmen. Ich konnte bei meinen langjährigen Versuchen niemals einen Unterschied im Verhalten von Tauben und Hühnern feststellen, weder in bezug auf das Entstehen der Krankheit, noch auf Heilung.

Gehirn [5] und Zitronensaft [6] isoliert resp. nachgewiesen werden. Alle diese Substanzen zeigten einen nahezu gleichen Schmelzpunkt und gleiche Eigenschaften. Zu jener Zeit scheiterte die weitere Untersuchung an dem Umstand, dass die erhaltene Ausbeute minimal war. Es wurde deswegen versucht, der Lösung der Frage auf einem anderen Wege näher zu kommen. Da die Substanzen offenbar zu den Pyrimidinbasen in naher Beziehung stehen, wurde die Wirkung von nahezu allen bekannten Purin- und Pyrimidinsubstanzen auf kranke Tauben untersucht [7]. Doch konnte hier niemals eine volle Heilwirkung festgestellt werden, obwohl die meisten Präparate eine sehr auffallende lebensverlängernde Wirkung zeigten, für die vorläufig noch keine ausreichende Erklärung vorhanden ist. Es zeigte sich, dass die wirksame Substanz offenbar zu einer neuen Gruppe von Substanzen zuzuzählen ist, der der Name Vitamine gegeben wurde, um die Wichtigkeit derselben für vitale Prozesse zum Ausdruck zu bringen. Diese Substanzen enthalten den Stickstoff in einer Bindung, die offenbar bis jetzt noch nicht in der Natur angetroffen ist, und welche der Tierkörper nicht imstande ist, aufzubauen und deshalb schon fertig geliefert aus dem Pflanzenreich beziehen muss.

Inzwischen ist von Suzuki, Shimamura und Odake [8] eine Arbeit über die Fraktionierung der Reiskleie veröffentlicht worden, die teilweise sehr an die von mir benutzte Methode erinnert. Diese sehr gut durchgeführte Arbeit ergab die Isolierung der Nikotinsäure, die, wie wir später sehen werden, vermutlich eine gewisse Bedeutung für unsere Frage besitzt. Ausserdem muss hervorgehoben werden, dass obwohl Nikotinsäurederivate in der Natur aufgefunden worden sind, die Nikotinsäure selbst zum ersten Male aus Naturprodukten dargestellt wurde. Diese Autoren berichten ausserdem über die Isolierung des Vitamins als Pikrat. Es gelang mir bisher nicht, diese Angaben zu bestätigen. Da die Wirkung nur auf zwei Tauben geprüft wurde, und da weitere Angaben über die Reinheit dieser Substanz fehlen, müssen wir hier die weitere Mitteilung der Autoren abwarten, bevor wir uns weiter darüber äussern können. Moore und Mitarbeiter [9] konnten ebenfalls das Vorhandensein der Vitamine in der Hefe, Cooper [10] im Fleisch bestätigen.

Bei der Neuaufnahme der Hefebearbeitung gelang es mir vor kurzem [11], neue Tatsachen zu gewinnen, die wohl geeignet sind, ein neues Licht in dieses Gebiet zu bringen. Es ist immer mehr für mich die Wahrscheinlichkeit zutage getreten, dass an der geringen Ausbeute der Vitamine unsere chemischen Methoden die Schuld tragen. Ich konnte mich

überzeugen, dass wenn man mit einem grösseren Ausgangsmaterial arbeitet, die Ausbeute nicht proportionell zunimmt. Wir müssen uns deshalb nach neuen Methoden umsehen, die uns gestatten werden, der Zersetzung des Vitamins, für die ich jetzt tatsächliche Beweise in Händen habe, vorzubeugen. Diese Methode würde nicht nur für die Beriberifrage von Nutzen sein, sondern sie würde uns gestatten, auch andere noch weit mehr zersetzliche Vitamine, wie das Antiskorbut-vitamin, sowie die Wachstums substanz zu isolieren.

Bei der Verarbeitung von 100 kg Trockenhefe nach dem schon früher angegebenen Verfahren gelang es mir, aus der Vitaminfraktion eine kristallinische Substanz zu isolieren. Die Substanz sah chemisch einheitlich aus, schmolz bei 210° und heilte kranke Tauben in Mengen von 4—8 mg, subkutan verabreicht, vollständig in 2—3 Stunden. Die Resultate erinnern sehr an die Angaben, welche Fühner [12] über die aus der Hypophyse isolierten Substanzen machte. Auch hier wurde in erster Linie ein kristallinisches Produkt erhalten, das die volle Wirksamkeit der Extrakte dieser Drüse aufwies. Bei einer späteren Reinigung aber gelang es, dieses Produkt in vier verschiedene Substanzen zu zerlegen. Ähnliche Resultate wurden auch in meinem Falle erhalten. Es liess sich nämlich die ursprünglich erhaltene Substanz in drei chemisch reine Produkte zerlegen, nämlich [13]:

1. Smp. 229 (korr.) $C_{24}H_{19}O_9N_5$.
2. Smp. 222 (unk.) $C_{29}H_{23}O_6N_5$.
3. Smp. 235 $C_6H_5O_2N$. Nikotinsäure.

Die Vitaminfraktion aus Reiskleie liess sich auf analoge Weise vorläufig in zwei Substanzen zerlegen:

1. Smp. 233 $C_{26}H_{20}O_9N_5$.
2. Smp. 235 $C_6H_5O_2N$. Nikotinsäure.

Bei den Tierversuchen zeigte sich, dass keine der drei Substanzen allein verabreicht eine nennenswerte Wirkung aufweist. Wird dagegen die Substanz $C_{24}H_{19}O_9N_5$ und Nikotinsäure zusammen und zwar in geringerer Menge, wie die einzelnen Substanzen, subkutan eingeführt, so konnte eine frappante Heilwirkung erzielt werden. Da die einzelnen Substanzen allein keine Heilwirkung besitzen, so kann der Vorwurf, dass es sich nur um eine Adsorption der wirksamen Substanz handelt, nicht aufrecht erhalten werden. Die Wirkung der Substanzen aus Hefe lässt sich gut aus der beigegebenen Tabelle ersehen²⁾.

²⁾ Die Angaben von Barsickow (Biochem. Zeitschr., 48, 418, 1913), dass das Hefepräparat „Cerolin“ von Böhlinger & Söhne (Alkoholextrakt aus Hefe) für beriberikranke Tauben unwirksam ist, erwies sich nach meinen Versuchen als irrig.

Nummer des Tieres	Verabfolgte Substanz	Dosis	Wirkung	Ueberlebend
1	Rohkrystallisation	4 mg	Heilung in 3 Stunden	4 Tage
2	"	8 "	" " 2 "	6 "
3	"	4 "	" " 3 "	4 "
4	"	8 "	" " 3 "	4 "
5	Substanz I	8 "	Part. Heil. in 7 Stdn.	3 "
6	"	4 "	Besserung	4 "
7	"	2 "	Besserung einige Stdn.	3 "
8	Subst. I u. Nikotins.	5 u. 2 mg	Heilung in 2 1/2 Stdn.	4 "
9	"	4 " 2 "	" " 3 "	5 "
10	"	3 " 2 "	" " 2 "	6 "
11	"	4 " 2 "	" " 2 1/2 "	7 "
12	"	3 " 2 "	" " 4 "	4 "
13	Nikotinsäure	1 cg	Kleine Besserung	2 "
14	"	5 mg	Vorübergehende Bess.	3 "
15	"	4 "	Kein Effekt	2 "
16	"	4 "	Besserung	1 Tag
17	Substanz II	5 "	Keine Wirkung	—
18	"	5 "	"	1 Tag
19	Die Mischung d. 3 Subst.	1 cg	"	—
20	"	1 "	Zeitweise Besserung	2 Tage
21	"	1 "	"	1 Tag

Es zeigte sich ferner, dass auch die vorhergehenden Fällungen vitaminhaltig waren; diese Fraktionen werden jetzt bearbeitet. An diese Fraktionierung knüpft sich unmittelbar eine Beobachtung, die, meiner Meinung nach, für das weitere Studium der Beriberifrage von gewisser Bedeutung ist. Es konnte nämlich konstatiert werden, dass, während die rohe, kristallisierte Vitaminfraktion, wenigstens qualitativ, wenn nicht quantitativ, dieselbe heilende Wirkung hat, wie die Originalextrakte, die daraus isolierten rein chemischen Substanzen schon etwas an der Wirksamkeit einbüßen, eine Beobachtung, die übrigens bei der Fraktionierung der Schilddrüsenextrakte, der Nebenschilddrüsen- und Hypophysenextrakte schon oftmals gemacht wurde. Es wurde nun gefunden, dass Hand in Hand mit dem Einbüßen in der Wirksamkeit der Vitaminpräparate, eine Farbenreaktion verschwindet, die von Folin und Macallum [14] für Harnsäure beschrieben worden ist, nämlich die Blaufärbung mit Phosphorwolframsäure in alkalischer Lösung. Ich bin nun mit Macallum [15] diesem Phänomen nachgegangen und zu folgenden Ergebnis gelangt.

Wir haben zuerst untersucht, welche Substanzklassen diese Reaktion liefern. Wir haben hier gefunden, dass es ausser der Harnsäure sowie ihren Methylsubstitutionspro-

dukten und Alloxantin keine weiteren Substanzen gibt, die diese Reaktion aufweisen. Man kann dieselbe einigermaßen als spezifisch für gewisse Puringruppen betrachten. Eine weitere ebenfalls von den oben genannten Autoren aufgefunden Reaktion mit Phosphorwolframsäure und Phosphormolybdänsäure wird ausser von Purinderivaten auch von Tyrosinderivaten und gewissen Phenolen geliefert. Wir haben nun gefunden, dass alle Nahrungsmittel, die bekannterweise Vitamine enthalten, ohne Ausnahme bis jetzt die beiden Reaktionen zeigen; auch alle wirksamen Vitaminfraktionen zeigen dasselbe Verhalten. Nun habe ich bei der Fraktionierung der Hefe- und Reiskleieextrakte gefunden, dass beim Reinigungsprozess diese Farbenreaktionen zum grossen Teil oder gänzlich zum Verschwinden gebracht werden. Es handelt sich offenbar um sehr labile Substanzen; wir bemühen uns schon seit einiger Zeit, diese Substanzen zu isolieren. Wir stiessen dabei auf grosse Schwierigkeiten; erstens konnten wir kein passendes Fällungsmittel ausfindig machen und zweitens ist diese Substanz so labil, dass schon Spuren von Säure oder Alkali dieselbe vernichten. Nach zahlreichen Versuchen sind unsere Bemühungen endlich von Erfolg gekrönt³⁾. Wir werden hoffentlich nächstens über eine Methode mitteilen, die uns gestatten wird, diese höchst labilen Substanzen ohne Zersetzung zu gewinnen. Dieselbe Methode wird voraussichtlich für die Isolierung anderer labiler Substanzen, wie z. B. auf Fermente, Hormone usw. dienen können.

Was das Wesen der Beriberi anbelangt, so müssen wir gestehen, dass wir uns noch vollständig im Dunkeln befinden. Ich stehe vorläufig auf dem Standpunkt, dass ausser dem Fehlen der Vitamine in der Nahrung keine weitere primäre Ursache für die Entstehung dieser Krankheit verantwortlich ist. Ich halte diese Ursache für primär, weil durch Zufuhr von Vitaminen alle Krankheitserscheinungen prompt zurückgehen. Alle anderen Hypothesen, wie z. B. die Giftwirkung auf die Nerven, entbehren zur Zeit jeder experimentellen Grundlage. Bis jetzt wurde die fettige Degeneration der Nerven fast allgemein auf Giftwirkung zurückgeführt. Es ist jedoch sehr wahrscheinlich, dass die Nervendegeneration bei Beriberi auf einem anderen, noch ganz unbekannten Wege entsteht. Die ausserordentlich schnelle Wirkung des Vitamins lässt sich nur schwer als eine antitoxische auffassen. Auch ist es sicher, dass die Nervendegeneration sekundären Ursprungs ist, da nach erfolgter Heilung dieselbe histologisch nachweisbar ist, während funk-

³⁾ Die Arbeit wird demnächst veröffentlicht.

tionell die Nerventätigkeit eine normale wird. Es wird die Aufgabe von Physiologen und Pharmakologen sein, uns aufzuklären, worin die Bedeutung der Vitamine liegt. Wie ich schon oft betont habe, wissen wir bereits, dass die Vitamine zu den lebenswichtigen Substanzen gehören⁴⁾. Ich habe schon an anderer Stelle ausführlich mitgeteilt, dass das vollständige Entziehen der Vitamine eine ganze Anzahl von Krankheiten zur Folge hat, die ich kurz als Avitaminosen, englisch „Deficiency Diseases“, bezeichnen möchte. Zu diesen Krankheiten kann Skorbut, die Möller-Barlow'sche Krankheit, höchstwahrscheinlich Pellagra und Rachitis [16] und andere mehr zugezählt werden. Ein dauerndes Ausbleiben der Vitamine in der Nahrung führt zum sicheren Tode. Das Studium der Beriberi hat den wichtigen Beweis erbracht, dass unsere Nahrung viel komplizierter ist, als bis vor kurzem geglaubt wurde. Bei der Fraktionierung grosser Mengen von Nahrungsmitteln stiess ich auf eine grosse Anzahl neuer Substanzen. Wir sind noch weit davon entfernt, die Zusammensetzung unserer Nahrung zu kennen. Die genauere Kenntnis der Bestandteile der pflanzlichen und tierischen Gewebe und besonders der Substanzgruppe, die ich als Vitamine zusammenfasse, wird uns erlauben, eine grössere Einsicht in die Zellfunktionen zu erlangen. Während die Substanzen, die sich in der Zelle in grösserer Menge befinden, schon bearbeitet worden sind, beginnt jetzt ein neues Kapitel, die Chemie der Mikrobestandteile der Nahrung, die sich durch ihre physiologische Bedeutung auszeichnen. Die Probleme, die sich unmittelbar an diese neuen Ideen anknüpfen, werden von mir an anderer Stelle ausführlich behandelt⁵⁾.

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⁴⁾ Vor kurzem erschien eine Arbeit von Abderhalden und Lampé (Zeitschr. f. d. ges. exp. Med. 1, 296, 1913), die die Existenz und Bedeutung der Vitamine in Frage stellt. Diese Versuche, die ich an anderer Stelle einer ausführlichen Kritik unterziehe, genügen keineswegs, um so schwerwiegende Schlüsse zu gestatten. Die Vitaminfraktion der Hefe oder der Reiskleie kann jederzeit auf ihre Wirkung und Bedeutung geprüft werden.

⁵⁾ Die Vitamine, ihre Bedeutung für die Physiologie und Pathologie, mit besonderer Berücksichtigung der Avitaminosen (Beriberi, Skorbut, Pellagra, Rachitis). Anhang: Wachstum und das Krebsproblem. Bei J. F. Bergmann, Wiesbaden, 1913. Im Erscheinen.

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XXXV. ON THE CHEMICAL NATURE OF SUBSTANCES FROM ALCOHOLIC EXTRACTS OF VARIOUS FOODSTUFFS WHICH GIVE A COLOUR REACTION WITH PHOSPHOTUNGSTIC AND PHOSPHOMOLYBDIC ACIDS. (PRELIMINARY COMMUNICATION.)

By CASIMIR FUNK, *Beit Memorial Research Fellow*, AND
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From the Biochemical Department, Lister Institute.

(Received May 22nd, 1913.)

Two colour reactions have recently been described, one with phosphotungstic acid for uric acid [Folin and Macallum, 1912], the other with phosphotungstic and phosphomolybdic acids for polyphenols [Folin and Denis, 1912], which they recommend for the determination of these substances. One of us previously observed during the fractionation of yeast and rice-polishings [Funk, 1913] that the vitamine-fraction constantly gave a blue coloration with both reagents, that the reaction gradually disappeared during the further purification of the products and at the same time that the curative power for polyneuritis in birds slightly diminished. As these reacting substances might be of some importance for the process of curing, we have tested a large number of compounds with both reagents, in order to obtain some information as to the chemical nature of these substances, and we have found that a certain number of them which occur in nature show the colour reactions.

With the uric acid reagent certain purine bodies and some tyrosine derivatives give a very strong reaction. The alcoholic extracts of different foodstuffs, especially those which are known to be rich in vitamins, give in general both reactions very markedly. The substances giving the reaction seem to pass into the phosphotungstic acid filtrate and we are proceeding to isolate them.

The reactions with purine and pyrimidine derivatives.

Substance	Uric acid reagent	Phenol reagent
Xanthine	-	++
Hypoxanthine	-	+
Paraxanthine	-	-
Guanine	-	++
Alloxantin	+++	+++
Alloxan	-	-
Thymine	-	-
Theophylline	-	-
3-Methyl-uric acid ..	+	++
7- " "	trace	++
Hydantoin	-	-
Uracil	-	-
Guanidine carbonate	-	-
Hydantoic acid	-	-
Allantoin	-	-
Adenine	-	-
Uridine	-	-
Guanosine	-	trace
Adenosine	-	-
Cytidine nitrate	-	-
Yeast nucleic acid	trace	trace
Thymonucleic acid	-	-

Tyrosine derivatives.

<i>l</i> -Tyrosine	-	+++
Nitro-tyrosine	-	-
3-4-Dihydroxyphenylalanine	+++	+++
2-Aminotyrosine	+++	+++
3-Aminotyrosine	++	+++
<i>l</i> -Tyrosine anhydride	-	-?
Glycyl- <i>l</i> -tyrosine	trace	++

We have also investigated a number of amino-acids, polypeptides and diketopiperazines, not including tryptophane, oxy-tryptophane and oxy-proline, all of which were entirely negative to both reagents.

Foodstuffs.

Ceridin (alcoholic extract of yeast) ...	trace	+
Zymnin	trace	+
Alcoholic extract of rice-polishings ...	+	++
Subs. $C_{24}H_{19}O_9N_5$ from vitamine-fraction of yeast	-	-
Subst. $C_{20}H_{23}O_9N_5$ from the same fraction ...	-	+
Nicotinic acid from yeast and rice ...	-	-
$C_{26}H_{20}O_9N_4$ from vitamine-fraction of rice ...	-	-
Alcoholic extract from caseinogen (crude) ...	+	+
Alcoholic extract of milk	+	+
Whey powder from milk	+	+
Filtrate from milk precipitated by acid ...	trace	+
Alcoholic extract of whey powder	trace	+
Cod liver oil and the aqueous extract ...	+	++
Alcoholic and aqueous extract of Rous' chicken sarcoma	trace	++

We see from the table that the reactions are very specific for purine derivatives and polyphenols and they may therefore serve as a guide as to what groups the substances giving the reactions may belong. As the reactions are very sensitive, it seems to us that they might be used to ascertain the purity of phosphatides and other substances like caseinogen which are prepared from foodstuffs, and for which up to the present we possess no standard of purity. This test has already been found very useful in the investigation of the vitamine-fraction.

The table for purine derivatives shows that a substitution of one hydrogen atom in the purine ring lessens or destroys the power to give the uric acid reaction. In the case of the phenol reagent this is also brought about when two hydrogen atoms are substituted. The colour reactions with tyrosine derivatives and alloxantin were remarkably stable as compared with the others.

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XXXVI. THE PRODUCTION OF ACETALDEHYDE DURING THE ANAEROBIC FERMENTATION OF GLUCOSE BY *BACILLUS COLI COMMUNIS* (ESCHERICH).

By EGERTON CHARLES GREY, 1851 *Exhibition Scholar*.

From the Biochemical Laboratory of the Lister Institute.

(Received June 8th, 1913.)

Acetaldehyde has long been known as a product of alcoholic fermentation by yeast [see Roeser, 1893]. It has likewise been found to be formed by the leaves of higher plants when their metabolism is restricted to anaerobic conditions [Kostytschew, 1913].

In view of this wide distribution, it was to be expected that acetaldehyde would be a product of bacterial fermentation. This is the case.

The quantity of acetaldehyde found by Roeser in various wine musts after a fermentation lasting from five to fifteen days, varied from 20–200 milligrams per litre of the fermented fluid. In the case of *B. coli communis* acting on glucose, one litre of fluid containing originally 15 grams of the sugar yielded in 15 days when half the sugar had been fermented 2·34 mg. of acetaldehyde, while, at the same time, 1·375 grams of alcohol had been produced. Since in the case of the true alcoholic fermentation quoted above, the quantity of alcohol formed was certainly twenty times that produced in the experiment with *B. coli communis*, it will be seen that the ratio of acetaldehyde to alcohol is of the same order in the two cases.

It is not at present settled, in the case of yeast fermentation, whether acetaldehyde is a primary product or whether it results from the oxidation of preformed alcohol, the following result is therefore significant in showing that the production of acetaldehyde by *B. coli communis* is related to the formation of alcohol, carbon dioxide, and acetic acid, rather than to the other products.

The experiment consisted in comparing the production of acetaldehyde from normal *B. coli communis* (Escherich) with that from a strain derived from the normal organism by selection with sodium chloracetate [see Penfold, 1911, and Harden and Penfold, 1912]. This derived strain had completely

lost the power of producing gas from carbohydrates. That it was closely related to the original organism was demonstrated by testing the agglutinating power of both the original and the derived organism towards a rabbit serum obtained after inoculating with the former. Both organisms agglutinated completely with the serum up to a dilution of one part of the serum in 25,600 of normal saline solution.

REACTIONS EMPLOYED FOR THE DETECTION AND DETERMINATION OF ACETALDEHYDE.

(1) *Rimini's reaction* [1898].

A few drops of diethylamine are added to a similar quantity of a solution of sodium nitroprusside. A blue colour appears which rapidly fades (2-3 minutes). If this solution be now diluted with 1-2 c.c. of water a pale yellow or yellow-green solution is obtained but if diluted with 1-2 c.c. of a solution containing acetaldehyde a blue colour results characteristic of this aldehyde. Though specific for acetaldehyde the reagent is not so delicate as that of Schiff, and the coloration is moreover less permanent.

(2) *Schiff's reagent*. This reagent was used for the determination of acetaldehyde.

It is important to make sure that no alkali is present in the fluid to be tested by Schiff's reagent since even the alkalinity of tap water produces a definite coloration. The maximum intensity of colour, by simple addition of alkali, was obtained with 0.1 c.c. normal KHO, added to 50 c.c. distilled water containing 0.25 c.c. Schiff's reagent. A greater concentration of alkali destroys the colour, which is regained on the addition of acid. The solution before examination was therefore always tested with phenolphthalein paper.

Acetone is said to give a coloration with Schiff's reagent, but this effect which is not produced for many hours cannot possibly be confused with the reaction for acetaldehyde in which case the coloration reaches a maximum in about twenty minutes and has very considerably faded in less than an hour. It is important to emphasise this fact, since Mendel [1911] states that he found acetone amongst the products of the action of *B. coli communis* and certain other organisms on glucose.

DETAILS OF THE DETERMINATION OF ACETALDEHYDE.

(1) About 750 c.c. of the fluid containing the liquid and solid products of fermentation (acetaldehyde, alcohol, and calcium salts of volatile and non-volatile acids etc.) was treated with oxalic acid in excess of that necessary for

the complete precipitation of the calcium. When the precipitate of calcium oxalate had settled, as much as possible of the supernatant liquor was removed with the aid of a siphon, and distilled. The first 400–500 c.c. of the distillate was made alkaline with barium hydroxide and redistilled, using a fractionating column, until 100 c.c. had been collected. In both distillations the receivers were well cooled by means of ice.

50 c.c. of this solution were used for the determination of the acetaldehyde, the method of procedure being an adaptation of that of Ryffel [1909].

(2) A standard solution of formaldehyde was prepared as described by Ryffel. The strength of this solution was determined by comparing the colour produced from it on the addition of Schiff's reagent, with that produced on adding the same quantity of the reagent to a solution of acetaldehyde prepared by the distillation of a known weight of lactic acid.

0.02494 gram lactic acid was converted into acetaldehyde. The intensity of colour produced on adding 0.5 c.c. Schiff's reagent to 100 c.c. of this acetaldehyde solution was 1.9 times that of the standard formaldehyde colour as measured by a Duboscq tintometer, 100 c.c. of the standard formaldehyde solution was therefore equivalent to $\frac{0.02494}{1.9} = 0.01312$ gram lactic acid.

Applying the ratio determined by Ryffel empirically for these conditions, viz.: 0.4 mg. formaldehyde = 3.435 mg. lactic acid = 1.765 mg. acetaldehyde, it follows that 100 c.c. of standard formaldehyde solution contains $\frac{0.4 \times 0.01312}{3.435} = 1.528$ mg. formaldehyde, and is equivalent to $\frac{1.528 \times 1.765}{0.4} = 6.471$ mg. acetaldehyde.

(3) 50 c.c. of the solution of formaldehyde described in paragraph (2) was diluted fivefold and compared with the distillate described paragraph (1), with the result:

$$\text{The ratio of } \frac{\text{standard}}{5 \times \text{distillate}} = \frac{1}{1.33}.$$

From this it was calculated that the total amount of acetaldehyde in the original litre of fermented fluid was 2.34 mg.

DISCUSSION OF RESULTS.

In order to demonstrate the relationship between the production of acetaldehyde and that of alcohol, carbon dioxide, hydrogen and acetic acid, the analyses of the products formed by the normal and the artificially selected strain are compared below, the results being calculated to 100 grams of the sugar.

	Products from glucose	
	Normal <i>B. coli communis</i> per cent.	Strain artificially selected by the chloracetate method per cent.
Hydrogen	0.42	Nil
Carbon dioxide	16.9	Nil
Alcohol	18.1	5.3
Acetic acid	18.5	10.8
Formic acid	9.7	11.1
Lactic acid	36.8	68.0
Succinic acid	0.7	0.8
Acetaldehyde	31.345 mg.	Nil
{ Glucose decomposed	7.465 gram	7.755
{ Acetaldehyde found	2.34 mg.	Nil

It will be seen that the artificially selected organism has (as in the case of the organism examined by Harden and Penfold), besides having lost the power of producing gas, also produced less alcohol and acetic acid. Coincidentally with these changes there has been an abolition or great reduction of the yield of acetaldehyde.

In the light of these results we may consider the question of the origin of the acetaldehyde.

If this substance were a secondary product formed by the oxidation of alcohol through the activity of oxidases concerned in the cell-growth as was suggested by Roeser for the case of yeast, then since there is a large excess of alcohol in both cases, the amount of acetaldehyde formed in the two experiments should be approximately the same, or at least, of the same order. Moreover in view of the fact that not only was air excluded from the fermentation flasks, but that in the experiment in which acetaldehyde was produced, 350 c.c. of hydrogen had been evolved, such an oxidation would seem unlikely to have occurred. The strongly reducing conditions of the experiment may be well seen from the experiment in which Harden attempted to employ asparagine as a source of nitrogen. This substance, he found, was completely reduced to ammonium succinate with corresponding diminution in the hydrogen evolved.

It may be suggested therefore that the production of acetaldehyde and part of the alcohol occur simultaneously. This would agree with the view held by Kostytschew [1912] that acetaldehyde is an intermediate product in alcoholic fermentation and would moreover suggest that part of the alcohol produced by the action of *B. coli communis* on glucose, passes through at least one of the same stages as that produced by the zymase of yeast.

From a consideration of the structure of glucose and mannitol, and the

fact that *B. coli communis* produces about twice as much alcohol from the latter as from the former, Harden [1901] suggested that the terminal group $-\text{CH}(\text{OH})\cdot\text{CH}_2\text{OH}$ which occurs twice in mannitol though only once in glucose, is related to the production of alcohol.



If then the final group $-\text{CHOH}\cdot\text{CH}_2\text{OH}$ conditions the production of alcohol, the corresponding group in glucose $-\text{CHOH}\cdot\text{CHO}$ might stand in the same relation to acetaldehyde.

Clearly since an accumulation of this product would be harmful to the organism natural selection would have evolved the organism capable of transforming the aldehyde by reduction into ethyl alcohol, or oxidation to acetic acid.

Further results will be shortly forthcoming with regard to the production of acetaldehyde from other substances allied to glucose.

SUMMARY.

(1) Acetaldehyde has been detected as a product of the action of *B. coli communis* on glucose, under anaerobic conditions.

(2) By artificial selection of *B. coli communis* by means of growth on sodium chloracetate, strains of the original organism have been obtained which produce either a greatly diminished amount of acetaldehyde or none at all.

(3) It has been found that the production of acetaldehyde is related to the formation of alcohol, carbon dioxide and hydrogen rather than to the other products. This has been ascertained by a comparison of the products formed by normal *B. coli communis* with those from an artificially selected strain produced by growth on agar containing sodium chloracetate.

(4) It is therefore suggested that acetaldehyde is a primary and not a secondary product of fermentation, and also that the process of alcohol formation by *B. coli communis* is in part analogous to the alcoholic fermentation set up by the zymase of yeast and to processes which occur in the leaves of higher plants.

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The Decomposition of Formates by Bacillus coli communis.

By EGERTON CHARLES GREY, 1851 Exhibition Scholar.

(Communicated by Dr. A. Harden, F.R.S. Received February 19,—Read
March 26, 1914.)

(From the Biochemical Department of the Lister Institute.)

Many observations have been made on the variability of gas production by intestinal bacteria under natural conditions (see Penfold (1911) and Arkwright (1913), where literature is quoted).

Penfold has found that by artificial selection of *Bacillus coli communis* in the presence of sodium chloroacetate, strains may be isolated which produce no gas from glucose and gas in lessened amount from mannitol, although in both cases acid is produced as with the normal organism. The writer has also shown that by artificial selection of *B. coli communis* by the chloroacetate method of Penfold, various stages between the original gas-producing and the selected non-gas-producing strain may be obtained, and the changes have been found to be associated in part with the disappearance of the enzyme which decomposes formic acid (1914). It was found that two kinds of artificially selected strains could be produced from the original strain of *B. coli communis*; one unable to decompose formic acid, and the other still able to bring about this decomposition provided glucose were present. The artificially selected organism, which could not decompose formates even in the presence of glucose, was likewise unable to produce gas from mannitol, whereas the organism which still retained the power of decomposing formates was also able to produce gas from mannitol, although it produced this gas in an amount approximately equal to one-half of that produced under the same conditions by the original *B. coli communis* from which it was derived. It seemed, therefore, likely that by a closer study of

the manner in which formic acid is decomposed by the natural and artificially selected varieties of intestinal bacilli it might be possible to gain information concerning the mechanism of the change brought about in the organism by growth on chloroacetate agar which leads to the selection of strains in some cases unable to decompose formic acid and in other cases unable to produce it to the same extent as the normal strains from which they have been derived.

It seemed also of importance to determine what use the decomposition of formic acid might be to the organism. Pakes and Jollyman (1901) and Harden (1901) have shown that *B. coli communis* is capable of decomposing a considerable amount of sodium formate, and that if a small quantity of glucose be added, the amount of hydrogen produced over and above that which could be derived from the glucose added is far greater than the amount produced in the absence of the sugar.

The writer has employed an artificially selected strain of *B. coli communis* obtained by the chloroacetate method; this strain produced in three days no gas from sodium formate peptone water, and only acid but no gas from glucose peptone water, but produced from a mixture of the two sufficient gas to fill the Durham gas tube (length 45 mm.) in 24 hours. The non-production of gas from sodium formate peptone water alone is due, not to the inability of the organism to decompose formic acid, but to the inhibitory action of the alkali due to the natural alkalinity of sodium formate; for if the sodium formate peptone water were acidified with sulphuric acid until the solution imparted a pink colour to litmus, it was found that a small quantity of gas was produced by growth of the artificially selected organism therein for two or three days.

Other sugars and polyhydric alcohols have been employed with similar results, which are discussed under Table II.

By a quantitative study of the decomposition by the bacillus in question of a mixture of glucose and calcium formate, the writer has been able to show that both the amount of glucose and that of formate decomposed is increased (Table III), and there can be little doubt that the formate and sugar are mutually helpful, in that the alkali produced by the decomposition of the former and the acid produced from the latter by neutralising one another maintain that approximately neutral condition of the medium which, as has been proved, is most favourable for the action of the organism.

EXPERIMENTAL.

The Examination of the Behaviour of Non-gas-producing Organisms towards Formates as a means of Deciding whether the Organism has been Derived from an Original Gas-producing Strain.

It has been mentioned above that by artificial selection of *B. coli communis* it is possible to obtain strains which do not produce gas from glucose, and that this phenomenon consists in part, in some cases, in a lessened power to decompose formic acid possessed by the selected organism. In the case of the strains examined by Penfold and Harden (1912) the power of decomposing formic acid was in all cases retained by the selected strains, and certain strains examined in the course of this work were found likewise to have retained this power. In the case of one strain, however, the power to decompose formic acid had been entirely lost. It may, therefore, be considered as probable that the strain incapable of decomposing formic acid represents a more advanced stage in the process of selection, and that, therefore, this type would be more permanent in character. Such indeed has proved to be the case, for while the strain which retains the power to decompose formic acid tends to revert in its properties to the parent organism as regards the production of gas from glucose, the other strain, which cannot decompose formic acid, shows no such tendency, although it has been frequently sub-cultured during the course of seven months.

In view of the fact that the more permanent non-gas-producing type of artificially selected strain is unable to decompose formic acid, it may be suggested that the same phenomenon might be exhibited by naturally occurring non-gas-producing organisms, and that in order to decide whether a strain which, at any particular time, does not produce gas has been recently derived from a gas-producing strain, an examination of its behaviour towards formic acid might be of crucial importance.

It frequently happens that organisms isolated from natural sources differ apparently only as regards the power to produce gas from carbohydrates and allied substances, and the question arises as to whether the one organism may have recently been derived from the other. Arkwright (1913), for example, has obtained varieties of *B. acidi lactici* differing in the aforesaid respect, both strains occurring in the same sample of urine, and he was also able to show that in certain cases the non-gas-producing strain could be trained to decompose sodium formate if grown for some time on a peptone water medium containing this salt. The writer has found that the power to produce gas from mannitol may, in some instances, be made to disappear by

simply allowing a broth culture of *B. coli communis* to remain unchanged for three months, or by growth of the gas-producing organism anaerobically in peptone solution containing mannitol in the presence of chalk for about one month. At the end of the period described, if a loopful of the culture be plated out on to agar, many of the colonies which grow at 37° will be found to produce no gas when inoculated into mannitol peptone water tubes. This change may be seen from Table I.

Table I.—The Disappearance from *B. coli communis* of the Power to Produce Gas from Mannitol by Continuous Growth of the Normal Organism in Unchanged Cultures.

History of the culture.	Production of gas.	
	Mannitol.	Glucose.
Normal <i>B. coli</i> recently isolated, average 46 normal strains	30 mm. gas	21·0 mm. gas.
The above-mentioned normal strains after being kept in unchanged broth 4 months, average 6 strains	25 "	22·0 "
Kept in unchanged broth 4 months, average 12 strains	12 "	20·0 "
" " 4 " " 12 "	5 "	18·5 "
" " 4 " " 8 "	2 "	20·5 "
" " 4 " " 9 "	Nil	21·0 "

The strains described in Table I, which did not produce gas from mannitol, were examined after growth on broth during several sub-cultures and were found not to produce gas from mannitol when inoculated from the broth tubes into mannitol peptone water. Thus the acquired character is inherited for a considerable time under these conditions. It will be seen from the foregoing table that no change has been brought about in the power to produce gas from glucose, and this is also true for dulcitol. Nevertheless, if by simple growth in peptone water *B. coli communis* yields a strain incapable of producing gas from mannitol, it would seem not unlikely that some similar process might, with time, lead to the disappearance of the power to produce gas from glucose, but such has not so far been observed.

In deciding whether an organism possesses the formic acid decomposing enzyme, which it is suggested here should be used as a criterion of a gas-producing organism, it is not convenient or sufficient to observe whether gas is produced from peptone water containing sodium formate. The test should be made with a mixture of sodium formate and glucose in such proportions that the sodium carbonate which will result from the decomposition of the formate will be approximately sufficient to neutralise the acid which will

be produced from the carbohydrate. A convenient mixture is 1·5 per cent. carbohydrate and 0·5 per cent. sodium formate in 1 per cent. peptone water. It will be found under these circumstances that whereas an organism may give only a few bubbles, or even no gas at all, from sodium formate peptone water alone, and none at all from glucose peptone water alone, the mixture may yield gas with great rapidity, so that in 20 hours a Durham tube may be completely filled. This increased gas production is due chiefly to the decomposition of the formate, but partly also to gas which may be produced from the sugar when the solution is maintained neutral, as will be described later.

This increased gas production from formates in the presence of carbohydrates is strikingly illustrated in the case of a selected strain of *B. coli communis* obtained by the chloroacetate method, as will be seen from the following table. The numbers represent millimetres of the tube occupied by gas in the Durham tubes of 45 mm. length.

Table II.—The Effect of Addition of Carbohydrates and Allied Substances on the Decomposition of Sodium Formate by an Artificially Selected Strain of *B. coli communis* producing only a Minute Quantity of Gas from Glucose.

Time.	Sodium formate.	Glucose.	Lactose.	Mannitol.	Dulcitol.	Sorbitol.	Glycerine.
(Concentration of the Sugar or Alcohol 2 per cent.)							
hours.							
12	Nil	Nil	Nil	Trace	Nil	Trace	Nil
24	"	"	"	11	"	12	"
36	"	Minute bubble	Minute bubble	23	"	25	"
60	"	No increase	0·5	No increase	"	No increase	Trace
84	"	"	2·0	"	3	"	1
108	"	"	3·0	"	30	"	1
132	"	"	3·0	"	37	"	1
Evolution of Gas from the above Carbohydrates and Alcohols after Admixture with Sodium Formate.							
(Carbohydrate or Alcohol 1·5 per cent., Sodium Formate 0·5 per cent.)							
12		12	4·0	Trace	Nil	2	Nil
24		Full	Full	10	"	10	"
36				34	"	Full	"
60				Full	"		5
84					5		7

The following facts should be noted in connection with the experiment described above:—

(1) The non-production of gas from formate peptone water alone was due, in part, to the natural alkalinity of the medium. To demonstrate this varying quantities of $N/10$ H_2SO_4 were added to a series of sodium formate peptone water tubes, which were then inoculated with a loopful of a broth culture of *B. coli communis*. It was found that in those tubes in which the reaction to litmus was nearest to neutral, there was a slight production of gas, whereas those which were distinctly alkaline or acid showed no gas at all.

(2) The manner in which the inoculation is made is also of importance. Several tubes of sodium formate peptone water were inoculated each with a loopful of a broth culture of *B. coli*, and another set of tubes were inoculated each with a loopful of an agar growth of the same organism. The former set of tubes produced no gas, the latter produced one-tenth of a Durham tube. This difference in the production of gas cannot be due simply to the size of the inoculation, for even when kept for 10 days the formate tubes inoculated from the original broth culture showed no production of gas. Probably, therefore, the bacillus when grown on agar contains more of the formic acid decomposing ferment than when grown in broth.

(3) The decomposition of sodium formate is not assisted in the same degree by mannitol as it is by glucose and the other sugars or by sorbitol, and it may be possible that this phenomenon is related to the fact already mentioned, that the power to produce gas from mannitol disappears from old broth cultures of *B. coli communis*, when these have remained unchanged for some months, and still more readily when the fluid contains mannitol and the products therefrom.

It should be noted also that, since less acid is produced from a hexahydric alcohol than from the same weight of a hexose when fermented by *B. coli communis*, the fact that the alcohol does not assist so well in the acceleration of the decomposition of the formate by the organism is in harmony with the view that it is the neutralisation of the medium by the acid produced by the carbohydrate or allied substance which is of assistance for the further decomposition of the formate.

The fact that in any particular experiment no gas may be produced from glucose peptone water is not a complete proof that an organism cannot produce gas at all from glucose, for the acid produced under circumstances in which no precaution is taken to neutralise the medium inhibits the decomposition of formic acid.

Quantitative Study of the Rate and Extent of Decomposition of Sodium Formate and Glucose by an Artificially Selected Non-gas-producing Strain of B. coli communis when grown on them either separately or together.

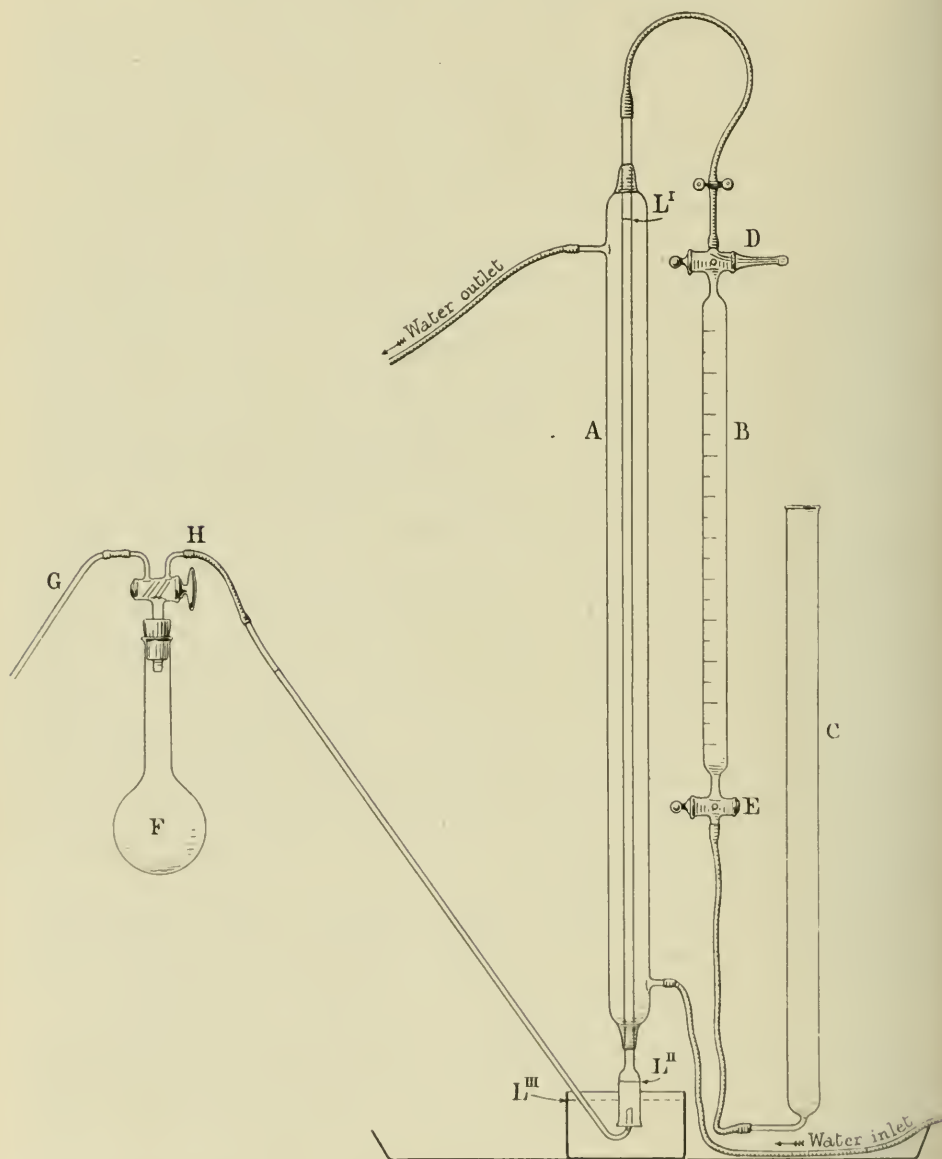
In order to determine the causes of the greatly increased gas production observed when *B. coli communis* was grown on a mixture of sodium formate and glucose, the change was followed quantitatively. For this purpose it was necessary to determine the weight of formic acid and glucose consumed in the reaction and the total carbon dioxide and acid produced, and also to measure the gas production from time to time. The method which was employed would be suitable for the examination of the decomposition of many other substances by bacteria, and it is therefore described in detail.

A quantity of 50 or 100 c.c. of 2 per cent. glucose in 1 per cent. peptone water is sterilised and inoculated with the organism. The cotton-wool plug, which should fit loosely, is pushed half-way down the neck of the flask, and the flask is connected with a Schiff's gas burette by means of a rubber stopper provided with a two-way tap. The burette, which is filled with mercury, is in connection with a reservoir for adjusting the pressure, as in the apparatus described by Harden, Thompson, and Young (1910). Before beginning the experiment, air may be removed from the flask by putting it in connection with the burette. On lowering the reservoir air passes into the burette. Nitrogen is then admitted to the flask by reversing the tap, and this process is repeated four or five times, when the oxygen will have been practically all removed. The flask is well immersed in a water-bath maintained at 37°. When it is desired to stop the reaction, the flask is removed from the water, and the contents are, after turning the two-way tap so as to put the flask in connection with the apparatus described below, carefully brought to the boil, the gas driven out displacing the mercury from the inner tube A (see figure).

Details of the Use of the Gas Collecting Apparatus.—The object of the apparatus is to collect all the gases which remain in the fermentation flask both free above the surface of the medium and dissolved in the fluid.

A is an ordinary Liebig's condenser set vertically and connected by a three-way tap D with a gas burette B accurately graduated. By putting D in connection with the pump or by raising the tube C, which must be filled with mercury, the mercury rises to fill B; the tap E is then closed. The tap D may now be reversed and mercury drawn up into the inner tube A from the reservoir L''' to the level L'. A circulation of water in the Liebig's condenser is not necessary for the condensation of the steam, but helps in keeping the temperature of the collected gas constant. To collect the gases

the flask F is heated carefully and the contents brought to the boil; the gas displaces the mercury from the inner tube A, and should the gas evolved be more than sufficient to fill A the tap D may be turned so as to connect



A and B, and the tap E turned so as to connect B and C, while C is lowered; the mercury in B falls with a corresponding rise of mercury in A.

The volume of the inner tube from a definite etched mark L'' to the tap

D, including the volume of the pressure tubing connecting A and B, having been previously determined, the total volume of evolved gases may be measured by raising the reservoir C, E being open, and D turned to connect A and B; the mercury then rises in B and falls in A, in which it is allowed to fall to the level L''. To correct for pressure an allowance may be made for the height of the mercury from the surface of the reservoir L''' to L'', but it is also quite convenient to lower the whole Liebig's condenser until L'' coincides with L'''. The volume of gases in the graduated tube B is then observed, and this volume added to that of the inner tube A. A sample of the gases may now be conveniently removed by lowering C. When B contains sufficient of the gases for analysis, the whole apparatus B-C may, if desired, be removed from its connection with A.

The apparatus has been described in detail because it is of use for the determination of all gases remaining in the fermentation flask. In the experiments recorded in the present communication, however, it was only of value to determine residual carbon dioxide.

Details of the Determinations.—The carbon dioxide boiled off from the solution, as described above, is measured in the usual way. The flask is now detached from the apparatus and the contents filtered from the deposit of chalk, and the filtrate and washings precipitated in hot solution with ammonium oxalate. The precipitate of calcium oxalate is used to estimate the calcium corresponding to the total acids produced during the fermentation, an allowance being made for the calcium in the peptone. The filtrate from the calcium oxalate is acidified with oxalic acid and distilled in steam, the distillate neutralised with deci-normal potash and evaporated to dryness; the residue is dissolved in about 50 c.c. of water, and the formic acid determined by the reduction of mercuric chloride. The residue from the steam distillation is made up to a definite volume, and an aliquot portion used for the determination of the residual sugar by Bertrand's method after the removal of peptone by Patein's mercuric nitrate reagent.

The results of the experiment are summarised in Table III.

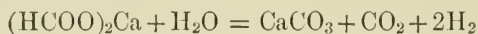
It will be seen from Table III that about ten times as much gas was produced by the selected strain of *B. coli communis* from calcium formate in the presence of glucose as was produced by it from calcium formate alone. The amount of sugar decomposed in the presence of calcium formate is considerably greater than in its absence, even when the medium is kept as far as possible neutral by chalk.

Table III.—Comparison of the Action of an Artificially Selected Strain of *B. coli communis* (Escherich) on Glucose alone; Glucose + Calcium Formate; Calcium Formate alone.

	Conditions of the experiment.			
	Glucose alone. Medium not neutralised during fermentation.	Glucose alone. Medium kept neutral by chalk.	Glucose + calcium formate + chalk.	Calcium formate alone.
Duration	99 hours	99 hours	120 hours	120 hours
Glucose before ...	3·385	1·6926	1·6926	None
„ after	3·2276	1·0628	None	„
„ consumed	0·1574	0·6098	1·6926	—
Formic acid before	None	None	0·5244	0·5244
„ „ after	0·0874	0·0249	0·0276	0·4988
„ „ con- sumed	—	—	0·4968	0·0256
CO ₂ total gas	42 c.c.	96 c.c.	291 c.c.	12 c.c.
CO ₂ from acids on chalk	41 „	90 „	161 „	—
CO ₂ from formate	—	—	119 „	12 c.c.
CO ₂ from sugar ...	—	6 c.c.	11 „	—

The medium contained in all cases 1 gm. peptone (Witte) in 100 c.c.

The actual carbon dioxide produced by the organism from calcium formate is in reality twice that actually evolved, for in the decomposition



it is clear that one-half of the CO₂ is retained in combination with the calcium.

These results bring out, therefore, very clearly one object which is attained by the decomposition of formates by these bacteria, viz.: that the organisms are thereby supplied with the best possible neutralising agent. For the formate by being decomposed into carbon dioxide and hydrogen virtually liberates alkali within the bacterial cytoplasm, and thus not only neutralises the medium, but also the bacteria themselves. Moreover the calcium formate being itself neutral possesses none of the disadvantages which would arise from the presence of even a slight excess of alkali. It would be difficult to devise a more efficient means for maintaining neutrality in this case. I would suggest the utilisation of sodium or calcium formate as a neutralising agent in working with those organisms capable of decomposing it, especially for solid media, with which the addition of dissolved alkali from time to time would be impracticable.

Summary and Conclusion.

(1) The power of *B. coli communis* to decompose formic acid varies considerably when the organism has been kept for some time on artificial media.

(2) The decomposition of formates is inhibited by a very small excess of either acid or alkali and, therefore, a greatly increased decomposition of formates results if glucose is added, since the acid produced from the sugar neutralises the alkali from the formate.

(3) A method and apparatus are described by which the decomposition of various substances by micro-organisms may be followed quantitatively requiring only 50-100 c.c. of the solution.

(4) It has been suggested that in place of a solution of sodium formate a mixture of sodium formate 0.5 per cent. and glucose 1.5 per cent. should be used as a test of a gas-producing strain, since by this means the production of gas from formate is greatly increased, and it is also suggested that the test could be used as a criterion as to whether an organism, which has been recently isolated from some natural source and produces no gas from glucose peptone water, may be regarded as having been recently derived from a gas-producing strain.

(5) It has been shown that formates may be conveniently used as neutralising agents, and that thereby the activity of gas-forming organisms may be considerably increased.

In conclusion I would express my thanks to Prof. Harden, F.R.S., for help and criticism.

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The Enzymes which are Concerned in the Decomposition of Glucose and Mannitol by Bacillus coli communis.

By EGERTON CHARLES GREY, 1851 Exhibition Scholar.

(Communicated by Dr. A. Harden, F.R.S. Received February 19,—Read March 26, 1914.)

(From the Biochemical Department of the Lister Institute.)

By the cultivation of bacteria in the presence of certain substances, for the most part toxic in character, it is possible to obtain strains in which the fermentative powers differ considerably from those of the parent organisms. As an example may be taken a variety of *B. coli communis* (Escherich) which was produced by the growth of that organism on agar containing sodium chloroacetate (see Penfold, 1911). This strain differed from the parent strain in that it now decomposed glucose with the production of acid but not of gas.

This result pointed to two possibilities; firstly the decomposition of glucose by the selected strain might be brought about by a set of ferments, which acted very differently from those of the normal strain responsible for the decomposition of the same substance, or secondly the primary cleavage products of glucose might be the same both from the original and the selected strain, and the difference between the action of the two might depend upon some secondary process, as for example the decomposition of formic acid, through which, as Pakes and Jollyman (1901) and Harden (1901) have shown, the carbon dioxide and hydrogen most probably arise.

It is obviously of great biological importance to know whether the changes brought about by growth on sodium chloroacetate result in any profound modification in the carbohydrate metabolism of the organism. It was, at the outset, considered most probable that those enzymes which were responsible for the cleavage of the glucose molecule into its primary products would be less likely to be lost than those which brought about the secondary changes.

It was hoped, therefore, that by a comparison of the products formed from glucose and mannitol by the normal organism with those produced from the same substances by the artificially modified strains it would be possible to determine how many different enzymes were concerned in the process.

If a number of products are formed by one enzyme the ratio which they bear to one another should not be altered by the process of selection, or conversely if on selection the ratio between any two substances is found to alter it may be taken as evidence that these two substances are not produced

by one enzyme, unless these two substances can replace one another to a certain extent owing to secondary reactions.

Isolation of the Organism.

B. coli communis (Escherich) was chosen for this work since the first observations made by Penfold (1911) on the disappearance of the gas-producing power by growth in the presence of sodium chloroacetate were made with this organism. It was found, however, that very many strains of *B. coli communis* could be isolated, showing not only differences in degree (which need not be considered here), but also of kind.

The organisms were isolated from human faeces in the ordinary way. A broth culture was made and from this bile salt cane-sugar neutral red agar plates were inoculated; after incubation for one or two days at 37°, a number of white colonies (cane-sugar non-fermenters) were removed to tubes containing lactose peptone water coloured with litmus, and provided with Durham gas tubes. Those tubes which on incubation produced acid and gas (lactose fermenters) were used to inoculate a series of tubes containing dulcitol peptone water. By these three operations organisms were obtained which according to MacConkey (1905) belonged to the *B. coli communis* (Escherich) group. The general characteristics of the four varieties which were found will be seen by reference to Table I.

Table I.—Characters of Strains of *B. coli communis* occurring together in Normal Faeces.

Motility.	Indole production.	Milk clotting.	Fermentation of sugars, etc.			
			Glucose.	Lactose.	Mannitol.	Cane-sugar.
1. Rapid	Strong	24 hrs.	A, G	A, G	A, G	Nil
2. Slight	Medium	24 „	A, G	A, G	A, G	Nil
3. Slight	Strong	5 days	A, G	A, G	A, G	Nil
4. Rapid	Nil	24 hrs.	A, G	A, G	A, G	Nil

A = acid. G = gas.

All the above strains were Gram negative, did not liquefy gelatin and did not give the Voges and Proskauer reaction. Very many examinations were made of the motility in from 3 to 10 hours' cultures.

The most striking difference is that between the rapidly motile No. 1 and the practically non-motile No. 2. These were chosen, therefore, for further study, since it seemed possible that the motile organism might differ considerably in its metabolism from that which was slightly motile.

The consideration of this relationship is, however, not one of the objects of the present communication.

It is important to note that the difference in motility between strain No. 1 and No. 2 is not merely one of degree but rather one of kind. It is, as a matter of fact, rather difficult to decide whether No. 2 is really motile at all, and only after concentrating the attention on one bacillus and observing its position from time to time in relation to an adjacent organism is it possible to decide that it really does exhibit a motion of translation. The strain was examined very many times in cultures from 3 to 12 hours' growth and at later periods, but no increase in the motility of this strain was ever observed. With the strain No. 1 the appearance is entirely different; in cultures of any age from 3 to 24 hours, rapid motility is readily observed. In cultures less than 9 or 10 hours' old the bacilli may be seen travelling with such rapidity that it is almost impossible to follow the course of any one particular bacillus. In young cultures (3 to 7 hours) the bacilli may be readily seen in long threads, in which the bacilli have not had time to separate. No such threads were obtained with strain No. 2.

The highly motile typical *B. coli communis* (Escherich) will be referred to as No. CI, and the feebly motile strain as No. CF.

Artificial Selection of Non-gas-producing Strains by Growth of B. coli communis (Escherich) on Agar containing Sodium Chloroacetate.

The technique of the chloroacetate method of selection has been described by Penfold (1911) and has been closely followed here. It has been found, however, that there is very considerable variation in the power of resistance to sodium chloroacetate, and also in the appearance of the chloroacetate agar plates inoculated with various strains of *B. coli* (Escherich). The nature of the changes brought about by growth in the presence of sodium chloroacetate will be discussed in a separate communication, and it must suffice to say here that the changes do not merely consist in the simple disappearance of the power to produce gas from glucose, but are, rather, of such a nature as to affect, to a greater or less extent, most of the enzymatic functions of the cell.

Some of the selected organisms are grown anaerobically only with great difficulty, and hence their chemical products cannot be readily investigated. Other strains show the property of spontaneously agglutinating and cannot, therefore, be very well shown to be derived from the original organism. In this work, only those selected strains which, by means of the agglutination test, could be demonstrated as related to the original organisms, have been employed for the examination of the decomposition products from glucose

and mannitol. Two varieties of selected organisms have been used—the one (CI selected) produced from the typical *B. coli* (Escherich) No. CI, like the organism of Harden and Penfold (1912), produced acid and no gas from glucose, while it still produced gas from mannitol, and also retained the power of decomposing formates in the presence of glucose (see Grey, 1914). The other (CF selected), obtained from the organism CF, now gave acid but no gas, either from glucose or from mannitol, and was also unable to decompose formates even in the presence of glucose. This second selected organism might perhaps be regarded as representing a further stage of selection than the first, but I have not found it possible so far to obtain from CI a strain comparable to CF (selected).

The Relationship between the Normal and the Artificially Selected Strains as established by the Agglutination Reaction.

The artificially selected strains (when made into an emulsion by the addition of normal saline to an agar growth) were found in many cases to agglutinate spontaneously. The freshly selected strain was therefore first plated out on plain agar and agar slopes made from several individual colonies. By this means it was found possible to obtain strains which did and strains which did not agglutinate spontaneously. The latter were then treated with rabbit serum containing the specific agglutinins for the normal strains CI and CF, and it was found that the serum obtained by inoculating a rabbit with normal CI agglutinated normal CI and the artificially selected

Table II.—Demonstration of the Relation between the Normal and Artificial Selected Strains (obtained by the Chloroacetate Method) by means of the Agglutination Test.

Bacterial emul- sion made with—	Dilution of the serum.									
	1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.	1/6400.	1/12800.	1/25600.	1/51200.
Serum obtained with CF normal.										
CF (normal)	+++	+++	++++	++++	+++	+++	+++	++	++	—
CF (selected)	+++	+++	++++	++++	+++	+++	+++	++	++	—
CI (normal)	—	—	—	—	—	—	—	—	—	—
CI (selected)	—	—	—	—	—	—	—	—	—	—
Serum obtained from CI normal.										
CI (normal)	+++	+++	+++	+++	+	—				
CI (selected)	+++	+++	+++	++	+	—				
CF (normal)	—	—	—	—	—					
CF (selected)	—	—	—	—	—					

strain derived from it, but did not agglutinate the strain CF. And, likewise, the serum obtained by inoculating a rabbit with the normal strain CF agglutinated the normal strain CF, and the selected strain derived from it (CF selected) up to a dilution of 1 : 25600, but did not produce the slightest agglutination with the normal or selected strain No. CI.* This may be seen from Table II.

Analytical Technique.

The methods of analysis described by Harden (1901) have been for the most part closely followed; certain slight modifications, however, have been introduced, which may be described here.

Volatile Acids.—In the steam distillate which is used for the determination of formic and acetic acid, the formic acid has been determined by the formation of mercurous chloride, and the acetic acid obtained by subtracting the amount of formic acid so found from the total acids determined previously by titration of the whole distillate with alkali, using phenolphthalein as indicator. Two errors are introduced here due to the presence of small amounts of carbonic acid and lactic acid in the distillate. The carbonic acid has, therefore, been estimated by barium hydroxide and the lactic acid by Ryffel's method. This estimation of lactic acid in the distillate becomes of importance when the amount of acetic acid is small.

The distillation to obtain the volatile acids was carried out in two stages. The first fraction was obtained without admitting steam, measured about 400 c.c., and contained the alcohol and part of the volatile acid. This fraction was titrated with standard baryta solution. A slight excess of baryta was then added, and the solution distilled with a fractionating column in order to remove the alcohol. The residual fluid now contained a granular precipitate of barium carbonate, which was removed by rapid filtration and titrated at the boiling point with N/10 H_2SO_4 . The barium hydroxide corresponding to the barium carbonate was deducted from that required to neutralise the first distillate. In this way an accurate correction may be made for the carbon dioxide dissolved in the distillate.

After removal of the first 400 c.c. steam was admitted, and the distillation continued until 100 c.c. of the distillate required only 0.1–0.2 c.c. of normal alkali for neutralisation. The total steam distillate usually measured about 2500 c.c. The distillate was neutralised with potash, united with the first fraction, and the whole evaporated to dryness. The residue was dissolved in 100 c.c. of water, and an aliquot portion used for the determination of

* The agglutinating sera were kindly prepared for me by Dr. J. A. Arkwright of this Institute, to whom my best thanks are due.

formic acid, another portion being used for lactic acid by Ryffel's method (1909).

The extent of the correction for carbonic acid and lactic acid in the distillate of volatile acids may be seen from the figures quoted in the table on p. 478.

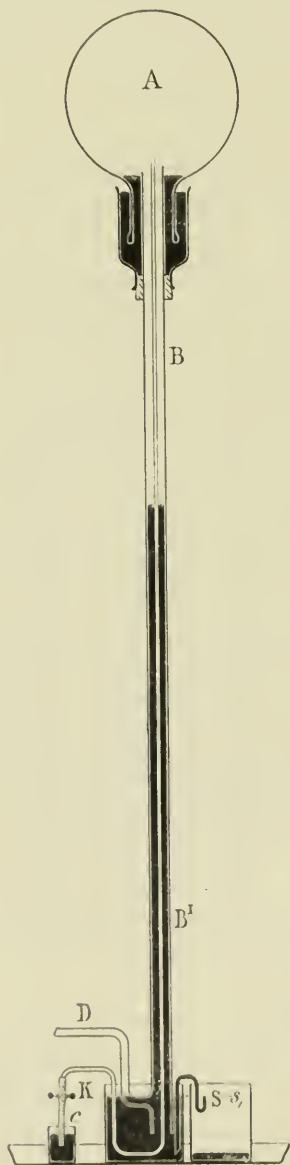
Collection of the Gas.

The carbon dioxide and hydrogen evolved were in some experiments collected in the apparatus of Harden, Thompson, and Young (1910); in other cases a simplified form of this apparatus was employed, which is here figured. The object of this modified form of apparatus is to dispense with all taps and to reduce the number of glass joints. The present form of apparatus has but one glass junction, and has also the advantage that when evacuated it can be sealed by allowing mercury to rise in the inner capillary tube through which the air has been pumped out of the flask. The arrangement for maintaining the pressure in the fermentation flask constant (by adjusting the level of the surface of the mercury in the reservoir automatically) is also of a simpler type.

The flask A is evacuated by means of the capillary tube, which passes up through the tube B in which mercury rises as the air is removed. The tube from the fermentation flask in the incubator is attached to D by a rubber junction. The gas in the fermentation flask is evolved under atmospheric pressure, this equalisation of the pressure in the flask with that of the atmosphere being effected by means of the S-shaped syphon (s), which is filled with mercury and automatically adjusts the level of the surface of the mercury in the mercury reservoir.

By plunging the rubber tube *c* beneath mercury and opening the clip K mercury may be allowed to rise in the capillary tube, and thus the flask A becomes completely sealed from the atmosphere.

The neck of the flask A may be plunged beneath wax. This substance



although quite effective, is somewhat troublesome to use, owing to shrinkage on cooling. A rubber stopper plunged beneath mercury is, on the whole, a simpler means of sealing off the flask.

Total volatile acid, c.c. normal potash.	Carbonic acid, c.c. normal baryta.	Lactic acid, c.c. normal (Ryffel's method).
48·5	1·44	1·68
38·2	1·0	1·1
34·3	0·3	0·8
51·5	1·0	1·0
41·1	1·3	0·6

Results of Analysis of the Decomposition Products of Glucose and Mannitol formed by the Action of the Normal and Selected Strains of B. coli communis.

Table III.

Typical <i>B. coli communis</i> (rapidly motile) No. CI.				
Product.	Normal.			Selected.
	per cent.	per cent.	Mean.	
On Glucose.				
CO ₂	14·90	14·74	14·82	2·25
H ₂	0·55	0·52	0·54	0·08
Formic	3·24	3·36	3·30	14·90
Acetic	14·10	12·91	13·00	5·69
Lactic	39·42	36·91	38·07	59·60
Succinic	3·60	4·60	4·20	5·50
Alcohol	12·83	11·02	11·93	4·90
			85·86	92·92
Ratio CO ₂ : H ₂ ...	1·23	1·29	1·26	1·28
On Mannitol.				
CO ₂	26·66	28·00	27·33	13·33
H ₂	1·04	1·06	1·05	0·64
Formic	7·21	7·56	7·39	17·48
Acetic	7·33	6·75	7·04	7·20
Lactic	22·82	26·27	24·55	19·95
Succinic	8·80	5·00	6·90	8·61
Alcohol	27·06	26·85	26·95	27·46
			101·21	94·67
Ratio CO ₂ : H ₂ ...	1·17	1·20	1·19	0·95

Table III—*continued.*

Variety <i>B. coli communis</i> (very slight motility) No. CF.				
	Glucose.		Mannitol.	
	Normal.	Selected.	Normal.	Selected.
	per cent.	per cent.	per cent.	per cent.
CO ₂	16·92	None	38·50	None
H ₂	0·42	None	1·45	None
Formic	9·73	11·80	1·57	32·50
Acetic	18·49	10·13	12·88	11·20
Lactic	36·83	62·00	7·48	15·84
Succinic	0·74	0·80	5·60	6·20
Alcohol	18·06	5·30	26·56	22·89
	101·19	90·03	94·04	88·63
Ratio CO ₂ : H ₂ ...	1·83		1·21	

These results may also be expressed as carbon atoms per molecule of glucose and mannitol respectively.

Table IV.

Product.	CI (rapidly motile).		CF (slightly motile).	
	Normal.	Selected.	Normal.	Selected.
	per cent.	per cent.	per cent.	per cent.
Glucose (carbon atoms per molecule).				
CO ₂	0·60	0·10	0·70	—
Formic	0·14	0·55	0·40	0·46
Acetic	0·81	0·34	1·11	0·61
Lactic	2·37	3·60	2·20	3·72
Succinic	0·22	0·27	0·04	0·04
Alcohol	0·90	0·39	1·41	0·40
	5·04	5·25	5·86	5·23
Mannitol (carbon atoms per molecule).				
CO ₂	1·09	0·54	1·58	—
Formic	0·28	0·69	0·06	1·27
Acetic	0·44	0·45	0·78	0·67
Lactic	1·48	1·20	0·45	0·95
Succinic	0·36	0·45	0·34	0·38
Alcohol	2·11	2·15	2·07	1·86
	5·76	5·48	5·28	5·13

Discussion of Results.

The most significant fact in connection with these results is that whereas in their action on glucose, the artificially selected strains of *B. coli communis* have been considerably modified, in their action on mannitol the only important change is the non-decomposition in the one case, and only partial decomposition in the other case, of formic acid into carbon dioxide and hydrogen.

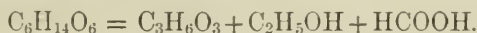
The results with mannitol present greater uniformity than those with glucose, and may be conveniently considered first. It will be seen that the ratio (alcohol + acetic acid)/2 : formic acid* is practically constant and almost equal to unity. Thus

$$\frac{2.55}{2 \times 1.37} = 0.93; \quad \frac{2.6}{2 \times 1.23} = 1.05; \quad \frac{2.85}{2 \times 1.64} = 0.87; \quad \frac{2.53}{2 \times 1.27} = 1.00.$$

This relationship also holds good for the earlier analyses of Harden (1901), and points to two conclusions—

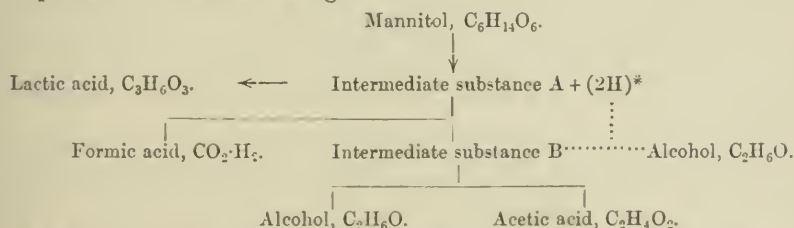
(1) Alcohol and acetic acid are probably derived from an intermediate substance common to them both, and they may therefore, to a certain extent, replace one another. (2) This intermediate substance from which alcohol and acetic acid are produced is itself formed in constant ratio to formic acid.

Lactic acid might be regarded as being formed directly from mannitol by the action of a special enzyme, but this could only occur if (a) hydrogen were evolved in excess of carbon dioxide, or (b) alcohol and formic acid were produced by the same enzyme which produced lactic acid, as, for example, in accordance with the equation



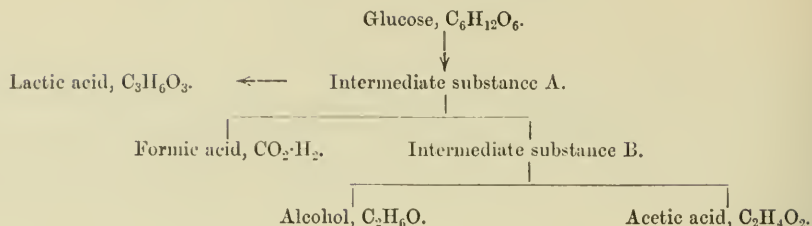
But if such a change as is represented by this equation were effected in one step by a single enzyme then, since the proportion of lactic acid actually produced is only one-third of that demanded by this equation (see Table IV), it would follow that there must be another origin for alcohol and formic acid.

The following hypothetical schemes are put forward to represent the decomposition of mannitol and glucose:—



* Hydrogen is here written as atomic hydrogen to indicate that it is intramolecular.

* Formic acid includes free carbon dioxide and hydrogen.



The intermediate substance A is unknown, but is postulated to account for the formation of lactic acid in such a way that the enzyme which produces lactic acid from glucose may also produce lactic acid from mannitol. The substance is probably related to pyruvic aldehyde.

The intermediate substance B from which it is suggested that alcohol and acetic acid are derived is probably acetaldehyde. This view is supported by the evidence that acetaldehyde may be detected among the products of decomposition of glucose by *B. coli communis* (Grey, 1913).

Two molecules of acetaldehyde might undergo the Cannizzaro reaction (Parnas, 1910) with the production of alcohol and acetic acid, thus



If this were the main change in the case of glucose, it would account for the production of alcohol and acetic acid in approximately equimolecular proportions.

Again, acetaldehyde might be reduced to alcohol as postulated by Kostytseff (1912) for alcoholic fermentation by yeast or directly oxidised.

In the case of mannitol this reduction might be of great importance. It is represented by the dotted lines in the scheme. And since, in this case the whole, or nearly the whole, of the hydrogen formed, along with the intermediate substance A, would be available for this purpose, the result would be the production of alcohol in large excess over that of acetic acid, which is actually observed.

While, however, the decomposition of mannitol and glucose may thus be represented as occurring along the same general lines, it is clear that some essential difference must exist between the mechanisms of the two reactions, or they would not be so differently affected by the process of selection on chloroacetate agar.

The simplest supposition is that this difference affects the production of formic acid and intermediate substance B, for artificially selected organisms produce these substances from glucose in greatly diminished amount, whereas from mannitol their production is not seriously altered.

While the exact nature of the difference in the two mechanisms must still

be a matter of conjecture, it may with some probability be supposed that it is connected with the presence in the products from mannitol of hydrogen available for reduction. It must be remembered that the two hydrogen atoms possessed by mannitol in excess of those present in glucose are only capable of reducing half the possible amount of B which could be produced from one molecule of mannitol. Hence, even if half the mannitol were converted into lactic acid, these extra hydrogen atoms could be completely taken up by B. As a matter of fact not more than one-quarter of the mannitol appears as lactic acid, so that a considerable part of B is reduced to alcohol and the remainder probably undergoes the same change as in glucose, forming equimolecular proportions of alcohol and acetic acid.

It must be noted that from the above considerations one would expect that the production of acetic acid from mannitol by the selected organism would be somewhat less than by the normal. In my figures, however, this is not demonstrated to be the case, but it must be remembered that the amount of acetic acid produced by the selected organism does not exceed that produced from glucose.

In the absence of more experimental results, however, it would be premature to discuss other possible origins of acetic acid.

In the scheme for the decomposition of mannitol the production of the excess of alcohol, as compared to the case of glucose, is represented as occurring through the agency of this extra hydrogen.

In the case of glucose, on the other hand, alcohol can only be produced if there be simultaneously the formation of some oxidation product, or in other words the hydrogen would have to be supplied by a reductase.

It should be remembered that the aldehydomutase of Cannizzaro which brings about the conversion in this case of two molecules of acetaldehyde into acetic acid and ethyl alcohol is in reality also a reductase, the acceptor for the oxygen being the same as the substance reduced. The essential difference between the two changes would then reside in the necessity for the co-operation of a reductase in the decomposition of glucose which would not be required to the same extent for that of mannitol.

In all other respects after the preliminary decomposition of the original molecule the two actions would then require exactly the same enzymes.

Considered dynamically, the reaction by which the intermediate substance A changes into formic acid and substance B occurs more rapidly with mannitol than with glucose, so that in the final products less lactic acid is formed in the case of mannitol than in the case of glucose.

This acceleration of the reaction in the case of mannitol by which intermediate substance A yields ultimately formic acid and alcohol as chief

products, may be due to the reduction of substance B to alcohol whereby it is removed from the sphere of the decomposition of A.

If, then, the reductase were to be diminished as the result of selection on chloroacetate agar, the removal of B from the sphere of decomposition of A would be slower. The decomposition of A into more of B and formic acid would therefore be specifically hindered, and as a result the production of lactic acid relatively increased.

This is what is actually observed. On the other hand the decomposition of mannitol would be unaffected, as is also found to be the case.

The view that the artificially selected strain produced by growth on chloroacetate agar is deficient in some reducing mechanism is further supported by the fact that many of these strains show diminished power of growing anaërobically. Moreover it might be expected that this method of selection would lead to the survival of a strain deficient in reductase, for a strain with a highly developed reducing mechanism would probably convert monochloroacetic acid to acetic acid with the liberation of hydrochloric acid, which would certainly not be of advantage to the organism. Such a process might therefore lead to the survival of the strain in which the reducing mechanism was poorly developed.

Summary and Conclusions.

Two artificially selected strains of *B. coli communis* obtained by growth of normal *B. coli communis* (Escherich) on agar containing sodium chloroacetate have been examined quantitatively as regards their action on glucose and mannitol. In both cases the selected strains produced from glucose, lactic acid in relatively greater, and alcohol, acetic and formic acid in relatively less, proportion than did the original strains from which they were derived, whereas from mannitol there was no diminution in the production of alcohol, acetic, and formic acid.

From these results it is inferred that the artificially selected strains have not lost the enzymes which bring about the final reaction in the production of alcohol and acetic acid, but that the process of artificial selection has led to an absence or diminution of the reducing mechanism of the cell so that some intermediate substance, from which formic acid and the precursor of alcohol and acetic acid are derived, cannot be readily decomposed.

In conclusion I wish to express my thanks to Prof. Harden, F.R.S., in whose laboratory this work has been done.

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XII. ON THE ACTION OF COAGULATING ENZYMES ON CASEINOGEN.

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Hammarsten [1872, 1874, 1877] first demonstrated that the rennin action on caseinogen was specific and independent of the action of the calcium salts. His explanation was that the caseinogen molecule was split up into a large molecule (Käse) and a smaller one (Molkeneiweiss). The "Käse" was rendered insoluble by the presence of soluble calcium salts and formed the clot. Since then little has been done to determine the chemistry of the clotting process, and our knowledge of this branch of the subject has up till recently been untouched by investigators.

The recent literature contains views which contradict the theory advanced by Hammarsten. Schryver [1913, 1 and 2] and Mellanby [1913] both consider that the rennin clot is probably a combination of enzyme and protein, and Schryver states definitely that rennin alone causes no proteoclastic change. Bosworth [1913] has found that the rennin does not split off any nitrogen from the caseinogen which remains in solution when the casein is precipitated by dilute acetic acid. The protein molecule has therefore undergone no cleavage into its components.

This, considered in connection with the results of his earlier work with van Slyke [1913], leads him to believe that the ferment breaks up the caseinogen molecule into two molecules of casein each half the size of the original molecule. In the case of basic calcium caseinogenate (containing 4 equivalents of calcium) the casein produced is soluble in water but is rendered insoluble by the presence of small quantities of calcium chloride. The caseinogenate containing two equivalents of calcium gives a casein insoluble in water¹.

¹ The English nomenclature—caseinogen for the unfermented protein and casein for the product of fermentation—is used throughout.

ENZYMES EMPLOYED.

The enzymes used were Witte's rennin powder 1:300,000, Grüber's trypsin, and a preparation made from the seeds of the *Withania coagulans* [Lea, 1884].

The rennin preparation was used in neutral aqueous suspension, was exceedingly active and fermented in a short time when the concentration was as low as 1:500,000. In the experimental work the concentration employed was 1 of rennin to 300,000 of caseinogen solution. The trypsin was used in aqueous suspension, in the proportion of 1 part of the solid preparation to 12,000 of caseinogen solution. The *Withania coagulans* enzyme was prepared by grinding up the seeds to a fine powder and extracting with 5 per cent. sodium chloride solution. Three volumes of absolute alcohol were added and the ferment precipitated. It was filtered, washed with absolute alcohol and dried at 37°. One gram of the powder thus obtained was made up to 100 cc. with 5 per cent. sodium chloride solution; 5 cc. of this solution were used to ferment 100 cc. of caseinogen solution. The trypsin and rennin contained no calcium or phosphorus estimable in three grams of the dried preparation, while the vegetable rennin contained a trace of phosphorus and no calcium.

PREPARATION AND PROPERTIES OF THE CASEINOGEN.

At the commencement of the investigation it was found that the commercial preparations of caseinogen, even when obtained from reliable sources, did not give satisfactory results. In fact the rennin had scarcely any appreciable effect on them. A modification of Hammarsten's method was finally adopted which gave satisfactory caseinogen preparations, Schryver's plan of drying in different grades of alcohol being employed.

The method was as follows:—skimmed milk containing 0.1 per cent. of fat was taken, diluted with five times its volume of distilled water and 0.1 per cent. of acetic acid added slowly, the whole being constantly agitated until the protein separated out. The caseinogen was then allowed to settle and the supernatant fluid syphoned off. The mixture of caseinogen and diluted milk serum was poured on a cheese cloth filter and the remaining fluid drained off. The caseinogen was then washed by mixing it with a volume of distilled water equal to the quantity of milk from which it was obtained and shaking thoroughly. When settled the water was again syphoned off and the caseinogen washed a second and third time with the same volume of distilled water. It was next redissolved by shaking with 0.2 per cent. sodium carbonate

solution and the precipitation and washing carried out as before, the solution, precipitation and washing being repeated at least twice after the original precipitation from milk. Finally it was ground up successively with 33 per cent., 66 per cent. and absolute alcohol and given two half-hour extractions in an agitator with ether, filtered and dried for two hours at 37°.

These preparations upon analysis were found to be completely free from fat and had a phosphorus content of 0.87–0.90 per cent. The ash from 10 g. contained no estimable calcium. Solutions were prepared by grinding the dried powder with moist calcium carbonate in a mortar, centrifuging and filtering to remove the excess of calcium carbonate, the caseinogen when treated in this way having a solubility of 19–20 cc. (5 cc. gave by Kjeldahl's method enough ammonia to neutralise this volume of N/10 acid). The solutions were opaque and milky in appearance, neutral to litmus and contained the amount of calcium necessary to form the basic caseinogenate. After treatment by rennin these solutions readily gave precipitates when about 5 per cent. by volume of N calcium chloride was added. This amount of calcium chloride alone with the original caseinogen solutions produced no precipitate from 0°–40°, but when the temperature was raised to 50°–60° a precipitate was formed. The precipitates produced by the action of enzyme and calcium chloride rapidly contracted to a small fraction of the volume of the solution, while those produced by calcium chloride and heat settled very slowly and did not shrink in volume.

COMPOSITION OF CASEINOGEN AND CASEIN.

The experiments were carried out as follows: 200 cc. portions of caseinogen solution were put into beakers and the enzymes added in the proportions already mentioned. Control experiments were made, using equal amounts of caseinogen solution and enzyme solution inactivated by boiling, and submitting these to the same conditions as those containing the active enzyme. The mixtures were placed in an incubator at 37° for one hour, after which 10 cc. N calcium chloride (5.5 g. per 100 cc.) were added both to controls and fermented preparations. In the case of the fermented solutions the precipitate at once came down. Precipitates from the controls were obtained by heating them to 55°. When the precipitates had settled the supernatant liquid was decanted and the precipitates thoroughly broken up and shaken vigorously with 500 cc. of 10 per cent. alcohol in a tall cylinder. When they had again settled the clear fluid was syphoned off and this process repeated three times.

The last washing was found to be free from chlorides. To inactivate the enzyme the precipitates were mixed with water and boiled for five minutes, the controls being similarly treated. All the precipitates were finally ground up with absolute alcohol and ether and dried at 37°.

The nitrogen, calcium, phosphorus ratios were determined as an index of composition, nitrogen being taken as the fixed unit of comparison. Phosphorus was estimated by Neumann's method and nitrogen by Kjeldahl's. Calcium was estimated by ashing the dried caseinogen or casein, dissolving the ash in hydrochloric acid and neutralising with ammonia until the phosphates were precipitated. These were redissolved by adding strong acetic acid, and the calcium precipitated as the oxalate and weighed as the sulphate. No phosphorus was precipitated by this method.

The results are given in the following tables.

I. *Rennin action.*

Caseinogen (controls)				Casein			
	N	P	Ca		N	P	Ca
1.	100	5.8	10.4	3.	100	5.65	9.60
2.	100	5.8	10.5	4.	100	5.60	9.53
				5.	100	5.45	9.64
				6.	100	5.65	9.66

II. *Trypsin action.*

Caseinogen (controls)				Casein			
	N	P	Ca		N	P	Ca
7.	100	5.54	12.41	9.	100	4.85	10.42
8.	100	5.50	12.15	10.	100	4.32	10.17
				11.	100	4.71	10.47

III. *Withania coagulans.*

Caseinogen (controls)				Casein			
	N	P	Ca		N	P	Ca
12.	100	5.58	12.58	14.	100	5.48	12.40
13.	100	5.61	12.62	15.	100	5.45	12.35

These series of analytical results demonstrate clearly that the enzyme action results in some proteoclastic change in the caseinogen molecule. The first series on rennin action was carried out with a preparation of caseinogen which was different from that used in the other two series and had a somewhat higher phosphorus content. Two criticisms of these results may be advanced—that it may not be possible to remove all the absorbed calcium from the casein and that boiling the precipitates to inactivate the enzyme may split off phosphorus, a possibility which Schryver points out. With regard to the first point it has been found that the final washing is free from chlorides and that a uniformly lower calcium content appears in casein throughout the series. To settle the second point caseinogen was taken,

fermented by rennin and calcium chloride added. The precipitate was filtered off and the N:P ratio was determined in the filtrate and compared with that given by a caseinogen solution to which equal amounts of calcium chloride and inactive enzyme had been added under the same conditions. In no case was the temperature higher than 37°, so that any difference observed must be due to enzyme action.

	Caseinogen (controls)			Filtrate from casein	
	N	P		N	P
1.	100	5.76	3.	100	12.2
2.	100	5.80	4.	100	12.0
			5.	100	10.7
			6.	100	13.1

This clearly demonstrates that the phosphorus cleavage was due to the rennin action alone and not to the boiling with water.

These figures for caseinogen are in agreement both in phosphorus and calcium content with most of the preparations of the basic salt quoted in the literature published on this subject.

On the other hand the casein shows a slightly lower phosphorus or calcium content relative to the nitrogen. These figures are not comparable with the phosphorus or calcium content of the acid salts of casein described by other investigators. Schryver finds that no change in the N:P ratio takes place when caseinogen is converted into casein, and van Slyke and Bosworth [1913] have prepared a basic caseinogenate and a caseinate from milk of the same phosphorus and calcium content.

The cleavage is most marked in the case of trypsin where from 12-20% of the phosphorus is split off, the action thus bearing a close resemblance to that exerted by this enzyme in alkaline solution [Plimmer and Bayliss, 1906]. The action is less marked in the case of the Withania enzyme.

ENZYMIC PRECIPITATION OF CASEINOGEN SOLUTIONS WITHOUT THE ADDITION OF CALCIUM SALTS.

Furthermore, caseinogen solutions after being subjected to ferment action give precipitates without addition of calcium chloride if the ferment be present in sufficient concentration. Caseinogen can thus be precipitated without the addition of calcium chloride in twenty-four hours by rennin when the latter is present in the proportion of 1 part of dried ferment to 1000 of solution. Trypsin in three to four hours also forms a precipitate in 1:5000 solution without the addition of calcium chloride. Withania coagulans also produced a small precipitate without addition of a soluble calcium salt in four

hours when one volume of ferment solution was added to five of caseinogen solution. Again, when the ferment was more dilute (rennin 1:100,000, trypsin 1:15,000, Withania 1:20) precipitates appeared when the temperature was raised from 45°–50° even when the ferments had acted only for twenty minutes and in the absence of added calcium salts. Controls containing the same concentrations of inactivated enzyme did not give these reactions.

NATURE OF THE CLEAVAGE PRODUCTS.

The filtrates from casein preparations were taken and examined to ascertain the nature of the nitrogen and phosphorus split off. Cathcart's precipitating mixture as used by Plimmer and Bayliss [1906] was added to casein solutions to precipitate the protein and its higher cleavage products, and analyses were made on aliquot parts of the filtrate. In the case of the casein produced by rennin action no soluble phosphorus or nitrogen was found. No inorganic phosphorus was produced by any of the three ferments.

100 cc. of caseinogen solution were taken containing 0.521 g. N and 0.0295 g. P. N:P = 100:5.6.

Produced from 100 cc. caseinogen solution and not precipitated by tannic acid			
	Soluble N	Soluble P	Ratio N:P
Trypsin action	1. 0.0203 g.	0.0033 g.	100:16.2
	2. 0.0084 g.	0.0015 g.	100:17.8
Withania action	3. 0.0028 g.	0.0006 g.	100:21.4
	4. 0.0022 g.	0.0009 g.	100:40.9

Trypsin as shown by these analyses gives the largest quantities of soluble nitrogen and phosphorus, and splits off some phosphorus group relatively rich in phosphorus, a N:P ratio considerably higher than the normal being obtained. In the case of the Withania enzyme the phosphorus is still higher in relation to the nitrogen but the total quantities of soluble nitrogen and phosphorus are very small. The amounts given above probably do not represent the total cleavage, some of the products in each case being presumably in a form precipitable by tannic acid.

As already mentioned the behaviour of trypsin in converting caseinogen into casein in neutral solution is somewhat like its proteoclastic action in alkaline solution, except that no inorganic phosphorus is produced as in alkaline solutions [Plimmer and Bayliss, 1906]. The effect of the trypsin used when allowed to act in alkaline solutions is shown by the following experiment.

Caseinogen solution and trypsin solution were taken in the same quantities as for the precipitation experiments, and enough alkali to make the concentration 0.2 per cent. NaOH added. This was incubated at 37° for three hours. Part was taken, the protein precipitated with dilute acetic acid and the inorganic phosphorus precipitated from the filtrate by magnesium citrate mixture, the phosphorus being estimated by Neumann's method in the phosphate precipitate. In another portion the soluble phosphorus was estimated as previously described.

100 cc. original solution.	Total P = 0.0268 g.
100 cc. solution after trypsin action.	Soluble P = 0.0150 g.
100 cc. solution after trypsin action.	Inorganic P = 0.0043 g.

THE IRREVERSIBILITY OF THE CHANGE.

Caseinogen when precipitated from solution in the presence of calcium chloride at 55°, washed with 10 per cent. alcohol, redispersed in caustic soda, again precipitated with acetic acid, washed and dissolved by calcium carbonate gave solutions not nearly as concentrated as those obtainable from the original material. These latter had concentrations of 17–19.5 cc., but after the above process products were obtained, the solubilities of which were only from 7–13 cc. These could readily be acted on by any of the enzymes and a characteristic casein precipitate obtained.

Casein after the above treatment had very low solubilities, ranging from 0.5–4 cc. and the solutions were transparent, and when submitted to further enzyme action did not yield products more readily precipitated by calcium chloride than themselves.

This confirms the observations of Hammarsten and Schryver on this point.

PRECIPITATION OF CASEINOGEN AND CASEIN BY ELECTROLYTES.

The precipitating action of a number of salts was tested on caseinogen and casein solutions prepared by means of calcium carbonate as described. 5 cc. of solutions exposed to ferment action for one hour and the same amount from controls containing inactivated ferment were diluted to 100 cc., boiled, cooled and 1 cc. quantities put in test tubes in which 1 cc. of the various salt solutions had been previously placed. The concentrations indicated in the tables are those of the salts in the final mixture. The mixtures were allowed to stand at room temperature for one hour; + indicates a precipitate.

TABLE I.

1. *Rennin action.*

Concentration of salt in final mixture	CaCl ₂		BaCl ₂		MgCl ₂		SrCl ₂	
	Casein- ogen	Casein	Casein- ogen	Casein	Casein- ogen	Casein	Casein- ogen	Casein
2N	-	-	-	-	-	-	-	-
N	-	+	-	+	-	-	-	-
N/2	-	+	-	+	-	-	-	+
N/4	-	+	-	+	-	+	-	+
N/10	-	+	-	+	-	+	-	+
N/20	-	+	-	+	-	+	-	+
N/40	-	+	-	+	-	+	-	-
N/80	-	-	-	-	-	-	-	-

2. *Trypsin action.*

2N	-	-	-	-	-	-	-	-
N	-	+	-	+	-	-	-	-
N/2	-	+	-	+	-	+	-	-
N/4	-	+	-	+	-	+	-	+
N/10	-	+	-	+	-	-	-	+
N/20	-	+	-	+	-	-	-	-
N/40	-	-	-	-	-	-	-	-
N/80	-	-	-	-	-	-	-	-

3. *Withania coagulans action.*

2N	-	-	-	-	-	-	-	-
N	-	-	-	-	-	-	-	-
N/2	-	+	-	+	-	-	-	-
N/4	-	+	-	+	-	-	-	+
N/10	-	+	-	+	-	+	-	+
N/20	-	+	-	+	-	+	-	-
N/40	-	-	-	-	-	-	-	-
N/80	-	-	-	-	-	-	-	-

Calcium and barium salts in the case of all the enzymes are most effective as precipitating agents. The ranges of the action of strontium and magnesium salts are similar, but are narrower than in the case of calcium and barium salts. This holds for all the enzymes with slight individual variations. In no case was caseinogen precipitated within the range employed.

In the case of the lanthanum salt (Table II, p. 98) the requisite concentration is very low, being N/800 for caseinogen. This is in accord with the findings of other workers, the trivalent ion being an exceedingly powerful one as a precipitating agent. Again the variation for the different enzymes is seen, but to a greater extent, especially for casein obtained by trypsin action.

The variation in the different caseins is probably due to the influence of the cleavage products present in the mixture which, as shown earlier, are different for each ferment.

TABLE II.

Precipitating action of lanthanum nitrate.

Concentration of salt in final mixture	Rennin action		Trypsin action		Withania action	
	Caseinogen	Casein	Caseinogen	Casein	Caseinogen	Casein
N/50	-	-	-	+	-	-
N/100	-	-	-	+	-	-
N/200	-	-	-	+	-	-
N/400	-	+	-	+	-	-
N/800	+	+	+	++	+	+
N/1600	-	++	-	++	-	++
N/3200	-	-	-	-	-	-

It is remarkable that both caseinogen and casein are precipitated by the same concentration of lanthanum nitrate, whereas they require very different concentrations of calcium, barium, strontium and magnesium salts.

The investigation of the precipitating action of rennin on caseinogen in milk is an extremely difficult problem, since here the reaction of the liquid and the presence of salts, carbohydrates and fats all play a part which may obscure the essential factors of the process. For this reason solutions of pure caseinogen have been used throughout the foregoing experiments. Another point which is of the greatest importance is the method of preparation of the caseinogen. The different methods used for this purpose probably account for much of the diversity of opinion which exists at present on the nature of the enzyme action. In our preparations from milk the use of sodium hydrate was avoided so as to escape the possibility of hydrolysing the protein. Van Slyke and Bosworth have used ammonia in the final stages of their method, allowing it to remain in contact with the caseinogen overnight. Their preparations have a very low ash content and the phosphorus content is the lowest yet recorded. This is due possibly to removal of phosphorus by the action of the alkali.

The foregoing results indicate that the substance precipitated by calcium chloride from solutions of caseinogen, which have been submitted to the action of rennin, trypsin or Withania enzyme, differ not only in precipitability by salts and solubility but also in chemical composition.

CONCLUSIONS.

(1) The conversion of caseinogen into casein by enzyme action is accompanied by the cleavage of nitrogen, phosphorus and calcium.

(2) Rennin action produces no soluble nitrogen or phosphorus. Trypsin splits off both soluble nitrogen and phosphorus, while the Withania enzyme

also produces soluble nitrogen and phosphorus but in smaller absolute quantities.

(3) The cleavage products are specific for each enzyme and it is to this difference of enzyme action that the variation in behaviour of the resulting casein is to be ascribed.

(4) The precipitation of calcium caseinate by soluble calcium salts is not due to any chemical combination with these.

(5) The caseinogen once exposed to enzyme action and redispersed cannot be rendered more precipitable by renewed enzyme action.

(6) If the enzyme be sufficiently concentrated, precipitates are obtained without the addition of calcium salts and the same thing occurs with more dilute enzyme solutions when the temperature is raised above 45°.

In conclusion we acknowledge our indebtedness to the Director of the Imperial Institute for the supply of seeds of *Withania coagulans* from which the enzyme was prepared.

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XIII. THE ENZYMES OF WASHED ZYMIN AND DRIED YEAST (LEBEDEFF). II. REDUCTASE.

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(Received January 13th, 1914.)

The reducing powers of yeast have long been known, and it was shown by Hahn that yeast juice and zymín possessed similar properties.

Recently the relation of the reducing ferment of yeast to the enzymes concerned in alcoholic fermentation has become of some interest, owing on the one hand to the theories of Neuberg, Kostytscheff and v. Lebedeff which postulate the reduction of acetaldehyde to ethyl alcohol as an essential step, and on the other hand to the experiments of Palladin [1908] who has found that during the fermentation of glucose by zymín the reducing action of the latter on sodium selenite and methylene blue is greatly diminished, and to those of Lvoff [1913, 1, 2, 3] who finds that during the reduction of methylene blue by dried yeast or maceration extract there is a correspondingly smaller fermentation of glucose, one molecule of methylene blue being equivalent to one molecule of glucose.

The reduction of selenite or methylene blue by living yeast is known [see Grüss, 1908] to be accelerated by the presence of glucose, and hence the conclusions of Palladin do not seem warranted without further examination.

In the following experiments the work of Palladin has been repeated and at the same time the effect of washing dried yeast and zymín upon the reducing power of the preparation has been ascertained.

THE REDUCING POWERS OF YEAST, ZYMIN AND DRIED YEAST TOWARDS SODIUM SELENITE.

Reduction of sodium selenite by living yeast. Influence of carbohydrates on the rate of reduction. The following mixtures were made up and incubated at 25°.

A.	2 g. yeast + 30 cc. 0.5 per cent. sodium selenite solution.
B. + 1 g. glucose.
C. + 1 g. galactose.
D. + 1 g. lactose.
E. + 1 g. arabinose.

The red colour due to metallic selenium appeared first in B and was quite marked in one hour. At this time there was only a faint reduction in all the other flasks. Two hours later the flasks all showed reduction, but that in B was very much more marked than in the others, which were all equally reduced. Hence the rate of reduction is increased by the presence of a fermentable sugar as found by Griess [1908]. The other sugars tried had no influence. A control experiment was carried out in which glucose was incubated with sodium selenite solution alone but no reduction took place. A second experiment in which other fermentable sugars, cane sugar and maltose, were employed, gave a similar result.

Reduction of sodium selenite by zymín. Similar experiments to those described above were next carried out, using zymín instead of living yeast. In no case was any acceleration produced by the presence of a fermentable sugar. Palladin [1908] states that the reduction in the case of zymín is greatly hindered and in some cases entirely inhibited under these circumstances. In the authors' experiments, however, such a retardation has only been observed when very high concentrations of sugar have been employed. With low concentrations (5 per cent.) of glucose little effect was produced.

These points are illustrated by the following experiments.

The following mixtures were made up and incubated at 25°.

						Concentration of glucose per 100 cc. in g.
A.	3 g. zymín + 10 cc. 1 % selenite + 10 cc. H ₂ O	0
B. + 9.4	+ 1 g. glucose	5
C. + 8.8	+ 2 g. ..	10
D. + 7	+ 5 g. ..	25
E. + 4	+ 10 g. ..	50

The total volume was thus the same in each case, while the concentration of glucose varied from 5 to 50 g. per 100 cc. After incubation for 60 minutes the reduction in A, B, C and D was well marked and was approximately equal in extent. In E reduction had just started but was much less pronounced than in the other flasks. Hence glucose in concentrations up to 25 per cent. had little influence on the rate of reduction. Palladin's explanation of his results was that in the presence of glucose the reductase was directly concerned in the alcoholic fermentation and was therefore not free to reduce the selenite.

In view of the different results obtained by the authors, with lower concentrations of glucose, it seemed desirable to examine to what extent fermentation was influenced by the presence of selenite, for which purpose the following experiments were carried out :

A. *Living Yeast.*

- (1) 2 g. yeast + 2 g. glucose + 20 cc. H_2O .
 (2) „ „ „ + 20 cc. 1 % sodium selenite.

The rate of fermentation of these mixtures at 25° was observed with the following results :

Duration of experiment, mins.	Total CO_2 evolved in cc.	
	1	2
	Yeast + glucose	Yeast + glucose + selenite
5	9.1	10.4
10	17.8	20.6
15	28.0	31.2
20	36.6	40.9
30	54.6	60.8

In the above experiment therefore the mixture containing the selenite gave a slightly higher rate of fermentation than the control.

Reduction in (2) was well marked after incubation for 20 minutes. With zymin on the other hand a very different result was obtained.

B. *Zymin.*

- (1) 2 g. zymin + 2 g. glucose + 20 cc. H_2O .
 (2) „ „ „ + 10 cc. 1 % selenite + 10 cc. H_2O .
 (3) „ „ „ + 20 cc. 1 % selenite.

Duration of experiment, mins.	Total CO_2 evolved in cc.		
	1	2	3
	No selenite	0.5 % selenite	1 % selenite
50	11.0	2.4	2.1
65	25.5	2.7	2.3
80	33.8	3.1	2.4
95	40.3	3.4	2.4
155	64.2	4.4	3.1

With zymin therefore the presence of even 0.5 per cent. sodium selenite almost entirely inhibited the fermentation and this result was confirmed by further experiments. Both (2) and (3) showed signs of reduction after 50 mins. and the production of selenium increased steadily during the experiment. As Palladin [1908] used concentrations of selenite varying from 2 to 5 per cent. it is improbable that alcoholic fermentation was proceeding at all in any of his experiments.

C. *Dried Yeast.* (Lebedeff.)

- (1) 2 g. dried yeast + 20 cc. H_2O + 2 g. glucose.
 (2) „ „ + 20 cc. 1 $\frac{0}{10}$ selenite + 2 g. glucose.
 (3) „ „ + 10 cc. 1 $\frac{0}{10}$ selenite + 10 cc. H_2O + 2 g. glucose.

Duration of experiment, mins.	Total CO_2 evolved in cc.		
	1 No selenite	2 1 $\frac{0}{10}$ selenite	3 0.5 $\frac{0}{10}$ selenite
35	18.8	0.6	0.9
95	36.6	1.0	1.5
155	55.6	1.5	1.9
380	98.6	3.8	3.8

Here, as with zymin, fermentation was almost entirely inhibited. Reduction was observed in (3) at the end of the experiment but not in (2).

The sample of dried yeast had a very low reducing power towards selenite as shown by the following experiment:

- (1) 2 g. dried yeast + 20 cc. 1 $\frac{0}{10}$ selenite.
 (2) „ „ + 20 cc. 1 $\frac{0}{10}$ selenite + 2 g. glucose.

These mixtures were incubated at 25° . After incubation for 4.5 hours no reduction could be detected in either. An hour later however reduction was visible in (2) and still later appeared in (1). These results with zymin and dried yeast cannot be explained on the ground of Lvoff's interpretation of his own experiments, according to which one molecule of glucose gives up two atoms of hydrogen to the reducible substance (in his case methylene blue), so that an amount of glucose equivalent to this escapes fermentation. In these experiments therefore in presence of 0.1 g. sodium selenite there should have been a deficit of about 29 cc. of CO_2 , whereas as a matter of fact the deficit in the case of dried yeast was about 95 cc. and in the case of zymin 64 cc., without any sign of fermentation setting in.

It may here be remarked that the conclusions drawn by Lvoff [1913, 2 and 3] from his experiments cannot at present be accepted. In all the experiments in which the fermentation was continued beyond the stage at which the methylene blue was completely reduced, the deficit of carbon dioxide and alcohol increased considerably with the time. This indicates that the inhibition of fermentation even in the earlier stages cannot be solely attributed to the deviation of hydrogen, and indeed makes it doubtful whether any of it can be attributed to this cause. Experiments on the change in the amount of sugar during the process which are being carried out by Lvoff and, it may be suggested, an investigation on the degree of inhibition produced with different concentrations of dried yeast or maceration extract are required before any definite conclusion can be legitimately drawn. Experiments VI

and VIII [Lvoff, 1913, 3, pp. 304-5] suggest that the effect varies considerably with the amount of dried yeast employed.

INACTIVATION OF DRIED YEAST AND ZYMIN BY WASHING.

It was observed that when zymin or dried yeast was washed several times with cold water and thus rendered incapable of fermenting sugar, it also lost its power of reducing methylene blue or sodium selenite.

It seemed therefore of interest to ascertain the cause of this loss of reducing power and also whether any substance capable of restoring it would at the same time restore the power of alcoholic fermentation. That the action is enzymic is shown by the fact that when dried yeast is boiled with water, the mixture does not reduce methylene blue. As a result it was found that the addition of certain aldehydes or of bouillon restored the reducing power but not the fermenting power, whilst the boiled washings restored both.

It seems probable therefore that washing removes some substance which acts as an acceptor for the oxygen activated during the reduction process and that the place of this can be taken by certain aldehydes or by some constituent of bouillon. The reducing enzyme of yeast therefore bears a close resemblance to that of potato juice recently investigated by Bach [1913, 1 and 2].

The zymin and dried yeast (Lebedeff) obtained from Schroder were washed in the manner previously described [Harden, 1913].

I. Washed zymin and sodium selenite.

5 g. zymin were washed and made to 60 cc.

1. 20 cc. zymin suspension + 20 cc. H_2O + 50 cc. 0.5 % sodium selenite.
2. " " " + 20 cc. boiled washings + 50 cc. 0.5 % sodium selenite.
3. " " " + 20 cc. H_2O + 50 cc. 0.5 % sodium selenite + 0.1 cc. formalin.
4. " boiled washings + 20 cc. H_2O + 50 cc. 0.5 % sodium selenite.

On incubation for 17 hours at 25° the only flask which showed reduction was (2), which contained washed zymin and boiled washings. The reducing power was not restored by the addition of formaldehyde (4).

II. Washed dried yeast and sodium selenite.

20 g. of dried yeast made to 100 cc.

1. 15 cc. yeast suspension + 10 cc. H_2O + 7 cc. 1 % selenite + 1 cc. toluene.
2. " " " + 10 cc. boiled washings + 7 cc. 1 % selenite + 1 cc. toluene.
3. " 0 + 15 cc. H_2O + 10 cc. boiled washings + 7 cc. 1 % selenite + 1 cc. toluene.

Reduction was marked in (2) after 3 hours at 25° and did not occur in either (1) or (3) in 17 hours.

III. Washed dried yeast and (a) Methylene blue, (b) Schardinger's reagent.

10 g. dried yeast washed and made to 100 cc.

1. 20 cc. yeast suspension + 20 cc. H_2O + 1 cc. methylene blue + 1 cc. toluene.
2. " " " + 20 cc. boiled washings + 1 cc. methylene blue + 1 cc. toluene.
3. " H_2O + 20 cc. boiled washings + 1 cc. methylene blue + 1 cc. toluene.
4. " yeast suspension + 20 cc. H_2O + 1 cc. Schardinger + 1 cc. toluene.
5. " " " + 20 cc. boiled washings + 1 cc. Schardinger + 1 cc. toluene.
6. " H_2O + 20 cc. boiled washings + 1 cc. Schardinger + 1 cc. toluene.

The methylene blue was made by diluting 5 cc. of a saturated alcoholic solution to 200 cc.; Schardinger's reagent by mixing 5 cc. of saturated alcoholic methylene blue with 5 cc. formalin and diluting to 200 cc.

After 19 hours reduction had occurred in (2) and (5) but in none of the others.

Hence formaldehyde does not restore the reducing power.

IV. Reducing power of inactivated yeast in presence of various substances.

A number of qualitative experiments were made to ascertain the efficacy of various substances in producing reduction when added to washed dried yeast and methylene blue.

The results may be tabulated as follows:

Reducing power restored	No change
Salicylaldehyde*	Quinol*
Benzaldehyde	<i>p</i> -Phenylene diamine
Anisaldehyde	Pyrogallol
Isovaleraldehyde	Pyruvic acid
Bouillon*	Citral
	Acetaldehyde

The substances marked with an asterisk were tested as to their capacity to restore the power of alcoholic fermentation to washed dried yeast in presence of a small concentration of phosphate, in all cases with negative results.

Methylene blue and sodium selenite were also found to be inactive in this respect.

SUMMARY.

1. The presence of a fermentable sugar favours the reduction of selenite by living yeast but has little influence on the reducing power of zymine unless the sugar is present in high concentration, when inhibition occurs.

2. Sodium selenite in concentration of 0.5 g. per 100 cc. almost totally inhibits the fermentation of glucose by zymine and dried yeast (10 g. per 100 cc. of 10 per cent. glucose solution).

3. When dried yeast or zymin is washed with cold water it loses its power of reducing methylene blue and sodium selenite.

4. Such washed preparations reduce methylene blue in presence of many aldehydes and of bouillon, but these do not restore to it the power of producing alcoholic fermentation.

5. Addition of the boiled washings to these washed preparations restores both the power of reducing methylene blue and of producing alcoholic fermentation.

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LXII. THE ENZYMATIC FORMATION OF POLY-SACCHARIDES BY YEAST PREPARATIONS.

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(Received Nov. 17th, 1913.)

Two reasons led to the institution of the following experiments. In an earlier paper [1904] the authors showed that the carbon dioxide evolved in the alcoholic fermentation of sugars by yeast juice was not equivalent to the sugar which disappeared from the solution, and ascribed this fact to the production of a hydrolysable compound of low reducing power. It was subsequently found that in alcoholic fermentation by yeast preparations a certain amount of hexosephosphate is formed, which has a lower reducing power than the sugar (about 75 per cent.) from which it is formed and would therefore in part account for the phenomenon.

In the second place it appears to follow from the authors' equations of fermentation [1908] that in the normal fermentation both of fructose and glucose half the sugar passes through the form of hexosephosphate, which is then hydrolysed. Since these two hexoses appear to yield the same hexosephosphate it would be expected that as the fermentation proceeded fructose and glucose alike would be partially converted into the same product of hydrolysis, and the rotations of their solutions should therefore tend to approximate to each other. The exact nature of the substance produced along with phosphoric acid by the hydrolysis of hexosephosphate in yeast juice is not definitely known, but all the evidence points towards its being fructose. Hence we should expect in all fermentations of glucose by yeast or yeast preparations a progressive conversion of glucose into fructose [compare Sutor, 1911]. This however has not been observed and experiments were therefore made on the subject, especially with the object of ascertaining whether the product of hydrolysis of the hexosephosphate underwent any secondary change, such as condensation to a polysaccharide.

The present experiments show that both from glucose and fructose one or more dextrorotatory polysaccharides are produced during alcoholic

fermentation by yeast preparations. The previous conclusion of the authors is therefore confirmed, but it is not yet settled whether the polysaccharide formation takes place at the expense of the glucose and fructose themselves or occurs indirectly as the result of the action of some enzyme on the product of hydrolysis of the hexosephosphate. Further investigations on this point are in progress.

It is well known that living yeast forms glycogen when brought into excess of sugar solution [see Pavy and Bywaters, 1907], and the behaviour of yeast preparations therefore indicates that the enzymes involved in this synthesis are probably, at least to some extent, still present and active. The isolation of a substance having the qualitative reactions of glycogen is a further confirmation of the observation of Cremer [1899] who found that in yeast juice free from glycogen a substance was slowly formed in the presence of sugar which gave the characteristic glycogen reactions.

EXPERIMENTAL.

Experiment 1. Three lots of 100 cc. maceration juice (from Schroder's dried Münchener yeast) were incubated at 25° with toluene until they had attained the temperature of the bath. To Nos. 1 and 2 were then added 25 cc. of a 40 per cent. solution of glucose and the evolution of carbon dioxide observed. At the same time No. 3 was boiled and cooled, and 25 cc. of the same glucose solution added.

In Nos. 1 and 2 a maximum rate of 21.6 cc. per 2 minutes was slowly attained, which then rapidly diminished until in 52 minutes a constant rate of 2.8 cc. per 2 minutes was reached. The initial high rate was due to the presence of free phosphate in the maceration juice, which was converted into hexosephosphate. During this period the total gas evolved was 293.6 cc. at room temperature and pressure. After 52 minutes No. 2 was boiled, whilst the fermentation in No. 1 was allowed to proceed for 17 hours 38 minutes, during which time 1458 cc. of CO₂ had been evolved. No. 1 was then boiled. The contents of all three flasks were then filtered, and the free phosphate estimated in an aliquot portion of each. The amount of glucose in each was determined by precipitating the proteins in aliquot portions with Patein's mercuric nitrate solution and estimating the glucose by means of Pavy's method.

The treatment with mercuric nitrate precipitates the hexosephosphate, so that in order to determine the amount of sugar used up allowance must be made for the quantity bound up in the form of hexosephosphate. This is

readily done since it has given rise to the CO_2 equivalent to the free phosphate present at the beginning, and can therefore be determined by subtracting the carbonic acid corresponding with the constant rate of fermentation from the total actually evolved, in the manner frequently described before.

Free phosphate in No. 3 = 1.110 g., in No. 2 = 0.130 g. and in No. 1 = 0.127 g. $\text{Mg}_2\text{P}_2\text{O}_7$, showing that the same quantity of sugar is still bound up as hexosephosphate in No. 1 as in No. 2.

CO_2 evolved up to the time when No. 2 was boiled (after				
52 mins. fermentation)	293.6 cc.
Rate = 2.8×26	72.8 "
Equivalent of phosphate	220.8 cc. or 201 cc. at N.T.P., i.e. 0.394 g.

This corresponds therefore to 0.79 g. glucose.

The amount of glucose converted into hexosephosphate may also be determined from the phosphate combined during the experiment. Phosphate bound up in No. 2 = $1.110 - 0.130 = 0.980$ g. $\text{Mg}_2\text{P}_2\text{O}_7$; equivalent therefore to $0.980 \times \frac{18.0}{122} = 0.795$ g. glucose.

The tables show the amount of glucose which cannot be accounted for as CO_2 and alcohol or as hexosephosphate, the glucose originally present being obtained from No. 3 by analysis.

Flask (2).

CO_2 evolved = 293.6 cc. or 267 cc. at N.T.P. = 0.53 g. equivalent to	1.06 g. glucose.
Glucose bound up as hexosephosphate	0.79 g. "
Total accounted for	1.85 g. "
Original glucose	9.98 g.
Final glucose	8.15 g.
Actual loss	1.83 g.

No disappearance of glucose was observed.

Flask (1).

CO_2 evolved = 1458 cc. or 1339 cc. at N.T.P. or 2.634 g. equivalent to	5.27 g. glucose.
Glucose bound up as hexosephosphate	0.79 g. "
Total accounted for	6.06 g. "
Original glucose	9.98 g.
Final glucose	0.91 g.
Actual loss	9.07 g.

Thus $9.07 - 6.06 = 3.01$ g. of glucose have disappeared.

The ratio between the reducing power and the optical rotation was determined in each mixture after the treatment with Patein's solution in

order to see if any active substance other than sugar were present. For the sake of convenience the rotation observed in a 400 mm. tube is compared with the reducing power determined by Pavy's method expressed as grams of glucose in 100 cc.

With pure glucose this ratio $\frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}} = +2.05$, whilst with pure fructose it is -4.03 .

These ratios were found to be

$$(1) + \frac{2.149}{0.120} = +17.91.$$

$$(2) + \frac{2.224}{1.08} = +2.06.$$

$$(3) + \frac{2.843}{1.32} = +2.15.$$

It is thus seen that in No. 1 some substance is present which has a much greater dextrorotatory power than has glucose, whereas in No. 2 all the rotatory power may be accounted for by the quantity of glucose present.

Experiment 2. A similar experiment was carried out with fructose (Kahlbaum), the following mixtures being employed:

- (1) 100 cc. maceration juice + 25 cc. 40 per cent. fructose + toluene.
- (2) 100 cc. „ „ + 25 cc. 40 per cent. „ „
- (3) 100 cc. „ „ + 25 cc. water + toluene.
- (4) 100 cc. „ „ + 25 cc. „ „

The juice in each case was kept in the bath until the temperature was attained, and the fructose and water then added. Nos. 1 and 3 were boiled immediately, whilst the others were incubated and the fermentation observed. In No. 2, a high phosphate rate of 53 cc. per 5 minutes was rapidly reached which then decreased as usual to a constant rate of 7.5 cc. per 5 minutes; at the end of 17 hours both Nos. 2 and 4 were boiled. During the first 70 minutes No. 2 had given off 352.5 cc. of carbon dioxide, the amount due to the phosphate thus being $352.5 - 14 \times 7.5 = 352.5 - 105 = 247.5$ cc.; the total evolved in the 17 hours was 1179.5 cc. at 762.8 mm. and 15°. No. 4 showed no fermentation at all.

A portion of each of the four filtered mixtures was treated with Patein's solution as in the last experiment, and the reducing power and rotations determined in an aliquot portion of the filtrate. The figures given are calculated for the total volume of the juice.

	1. Original mixture	2. After 17 hrs.	3. Original juice alone	4. Juice incubated alone
Rotation in 400 mm. tube	-30.81°	$+4.65^{\circ}$	-0.061°	-0.066°
Total sugar as g. glucose	9.31	1.10	0	0

Loss of sugar = (1) - (2) = 8.21 g.

CO₂ evolved = 1179.5 cc. at 762.8 mm. and 15° = 2.08 g.

CO₂ corresponding to fructose bound up as hexosephosphate = 247.5 cc. at 762.8 mm. and 15° = 0.41 g.

Total sugar accounted for as CO₂ and hexosephosphate therefore

$$= 2 \times 2.49 = 4.98 \text{ g.}$$

Sugar disappeared = 8.21 - 4.98 = 3.23 g.

The ratios of rotation to reduction expressed as before were also determined in the solution after treatment with Patein's solution and were found to be (1) - 4.14, (2) + 5.28; pure fructose = - 4.03.

It is thus seen that in this experiment a substance having a high dextro-rotation was formed, so that although all the fructose was not used up, the mixture had changed in rotation from laevorotatory to dextrorotatory, whilst a much larger proportion of the original fructose had disappeared than could be attributed to the fermentation.

In the solutions before treatment with Patein's solution the free phosphate was estimated and was found to be :

(1) 1.203 g. Mg₂P₂O₇.

(2) 0.174 g. „

(3) 1.210 g. „

(4) 1.208 g. „

From this it is seen that the phosphate and hence an equivalent portion of the sugar was still bound up as hexosephosphate at the end of the experiment (No. 2). These numbers serve as before as a check on the quantity of sugar which has been converted to hexosephosphate, viz. that amount corresponding to

$$1.203 - 0.174 = 1.029 \text{ g. Mg}_2\text{P}_2\text{O}_7 \text{ or } 1.029 \times \frac{186}{222} \text{ fructose} = 0.834.$$

Fructose calculated from equivalent of CO₂ as above = 0.41 × 2 = 0.82 g.

The mixtures before treatment with Patein's solution were tested with iodine solution; Nos. 1, 3, and 4 gave no colouration, whereas No. 2 gave a deep reddish brown colouration.

A portion of No. 2 treated with three volumes of alcohol gave a white precipitate, which was redissolved in water and again precipitated with alcohol. This last precipitate gave an opalescent solution in water which was precipitated by saturation with ammonium sulphate and gave a red colouration with iodine. The only difference which could be seen from the behaviour of glycogen was that it gave a somewhat different red colour with iodine.

The other solutions Nos. 1, 3, and 4 gave slight precipitates with alcohol, the aqueous solutions of which gave however no colouration with iodine.

It is thus seen that during the fermentation of fructose by maceration juice a dextrorotatory, glycogen-like substance is formed.

These results appear to us as already indicated to throw some light on the cause of the difference which exists between sugar fermented and carbon dioxide evolved, not only in the case of yeast preparations but also in that of living yeast. Euler and his colleagues in recent papers have argued from the existence of this difference between the amount of sugar actually removed by living yeast from a glucose solution and the amount equivalent to the CO_2 evolved, which he terms $\Delta - C$, that the hexose requires to undergo some change which renders it directly fermentable and that the difference $\Delta - C$ represents the amount which is in this intermediate condition. [Euler and Johansson, 1912; Euler and Berggren, 1912.] There seems however to be no good reason to suppose that Euler and Johansson's $\Delta - C$ cannot be accounted for by the well-known formation of glycogen which has been shown by Pavy and Bywaters [1907] to be of the order of magnitude required.

In Euler and Berggren's experiments on the effect of yeast extract in increasing both rate of fermentation and $\Delta - C$ [1912], no counts of yeast cells before and after the experiments were made. As the earliest observations were made after an hour at 15° – 18° and the experiments in some cases extended to over six hours (1 g. pressed yeast in 25 cc. of solution), the possibility of yeast growth must not be overlooked. This is still more probable in the cases in which only 0.25 g. of pressed yeast was taken and tested with yeast extract itself, various precipitates from yeast extract, and with sodium nucleinate or ammonium formate [1912, pp. 216, 217; Euler and Cassel, 1913], in a total volume of 40 cc.

An experiment made on this point showed that under similar conditions of concentration growth readily occurs at 25° . The yeast was added as 5 cc. of a suspension of 5 g. yeast in 100 cc. H_2O , i.e. 0.25 g. yeast.

(1) and (2) 5 cc. yeast suspension + 20 cc. of 20 per cent. glucose + 15 cc. H_2O .

(3) and (4) 5 cc. yeast suspension + 20 cc. of 20 per cent. glucose + 15 cc. yeast extract.

In 345 mins. the evolutions were respectively 61, 59.6, 146.3, 150 cc. At the close of this time the numbers of cells present per cc. were 68.7×10^6 , 68.5×10^6 , 105.25×10^6 , 98.8×10^6 .

Asparagine acts in a precisely similar manner, 0.25 g. added to 0.5 g. yeast in 30 cc. sugar solution increasing the evolution in 2 hrs. at 25° from 73.6 to 89.4 cc.

It therefore seems that the experiments in which Euler has shown the accelerating effect of yeast extract, sodium nucleinate, etc., on the action of living yeast require revision from this point of view.

The method of testing for a co-enzyme by the action of solutions on living yeast is moreover open to the criticism that the yeast cell is, if at all, only imperfectly permeable to the co-enzyme so that negative results would be of little value.

SUMMARY.

During the alcoholic fermentation of glucose and fructose by Lebedeff's maceration extract of dried yeast, dextrorotatory polysaccharides are produced, and it is to the formation of these that the difference between the sugar removed and that equivalent to the carbon dioxide evolved is principally to be attributed.

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XXVIII. THE ENZYMES OF WASHED ZYMIN AND DRIED YEAST (LEBEDEFF). III. PEROXYDASE, CATALASE, INVERTASE AND MALTASE.

BY ARTHUR HARDEN AND SYLVESTER SOLOMON ZILVA.

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PEROXYDASE.

On the assumption that hydrogen peroxide and peroxydase might possibly be capable of oxidising some degradation product of sugar, Bach [1906] submitted sugar to the simultaneous action of hydrogen peroxide, peroxydase and an enzyme capable of decomposing sugar. He chose for his experiments the fermenting enzyme of yeast and employed zymine. Being however of the opinion that yeast contains no peroxydase he made use in his investigations of a peroxydase prepared from horse-radish. Although unable to observe any of the oxidation processes the probability of which he had anticipated, he found that when the peroxydase used was previously boiled the amount of carbon dioxide evolved was invariably more than when it was not boiled. This led him to conclude that the presence of active peroxydase had a deterrent effect on the fermenting power of zymine.

The authors find that fresh English brewery yeast contains active peroxydase. When however the yeast is dried for 17 hours at a temperature of 37° it no longer gives the reaction. On washing the dried yeast however the presence of peroxydase can again be detected.

Dried Munich yeast (Schroder) behaves like dried English yeast and does not react for peroxydase when unwashed, but regains that activity on being washed. Whether the peroxydase in yeast has a deterrent effect on the fermentation the authors are at present not prepared to say. It is to be observed that Bach in his experiments used a peroxydase from an outside source (horse-radish) and the presence of an agent in that preparation that would have a retarding effect on fermentation and which could be destroyed by boiling is not impossible.

EXPERIMENTAL.

Experiment 1. Ten g. of pressed English brewery yeast were washed four times to remove adhering wort, etc., and suspended in 300 cc. of distilled water. The washing in this and all the other experiments was done by stirring up the yeast in pots with 120 cc. of distilled water for the first time and 100 cc. for all subsequent times and centrifuging. Into two small Erlenmeyer flasks were introduced:

Flask 1. 10 cc. of yeast suspension (equivalent to 0.1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 (10 per cent. dilution of 3 per cent. H_2O_2 neutralised to litmus).

Flask 2. 10 cc. of yeast suspension + 1 cc. 1 per cent. p-phenylenediamine.

Flask No. 1 showed the first sign of a colouration in one minute and assumed an intense violet colouration in three minutes.

Flask No. 2 remained unchanged.

It is evident from this experiment that English yeast contains an active peroxydase.

Experiment 2. A sample of the yeast used in the previous experiment was then dried for 17 hours at a temperature of 37° and one gram of the dried yeast suspended in 100 cc. of distilled water. Into two flasks were introduced:

Flask No. 1. 10 cc. of yeast suspension (0.1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 (10 per cent. dilution of 3 per cent. H_2O_2).

Flask No. 2. 10 cc. of yeast suspension + 1 cc. 1 per cent. p-phenylenediamine.

Although flask No. 1 after one minute assumed a slightly darker colour than the control flask No. 2, it did not show any violet colouration and the reaction was decidedly negative. The repetition of the above experiments gave similar results and we may conclude that drying inhibits the activity of the peroxydase in the yeast.

Experiment 3. Some dried English yeast was washed four times; this process removes soluble phosphates, the coenzyme of zymase and, doubtless, other substances. Into two flasks were introduced:

Flask No. 1. 10 cc. of washed yeast suspension (0.1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. dilute H_2O_2 .

Flask No. 2. 10 cc. of unwashed dried English yeast suspension (0.1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. dilute H_2O_2 .

Flask No. 1 showed a colouration after 2 minutes. Flask No. 2 showed no colouration after 5 minutes. This experiment shows that the activity of the dried yeast can be restored by washing.

Experiment 4. 5 g. of dried English yeast were washed four times. The first washings amounted to 32 cc. Six Erlenmeyer flasks were taken and in each were placed 10 cc. of washed yeast suspension (0.1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. dilute H_2O_2 .

To flask No. 1 nothing was added.

„ No. 2 0.6 cc. of the washings was added.

„ No. 3 1 cc. „ „ „

„ No. 4 1.5 cc. „ „ were added.

„ No. 5 5 cc. „ „ „

„ No. 6 5 cc. of the neutralised washings were added.

No. 1 showed a colouration after one minute, No. 2, No. 3 and No. 4 after 3, 4 and 4 minutes respectively. No. 5 and No. 6 showed no colouration after 15 minutes. This experiment shows that the addition of the washings inhibits the action of the peroxydase in yeast.

Experiments similar to Nos. 3 and 4 were carried out with dried Munich yeast (Schroder) and zymin, and the results obtained were identical with those obtained with English dried yeast. It is however to be observed that various samples of zymin gave reactions of different intensity.

It is evident from these results that after the yeast cell has been dried or treated with acetone washing removes some substance which has an inhibiting effect on the peroxydase of the yeast. The washings not only inhibit the action of the peroxydase in yeast but also exert a deterrent effect on that of milk as shown by Experiment 5.

Experiment 5. Into two flasks were introduced:

Flask No. 1. 1 cc. dilute H_2O_2 + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. of milk + 5 cc. of water.

Flask No. 2. 1 cc. dilute H_2O_2 + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. of milk + 3 cc. of water + 2 cc. of washings from dried Munich yeast.

Flask No. 1 showed a colouration immediately. Flask No. 2 showed no colouration after 5 minutes.

It was further observed that the action of the inhibiting agent seems to increase in a higher proportion than that of the peroxydase as the amount of yeast is increased.

The following experiment illustrates this point.

Experiment 6. Flask No. 1. 0.05 g. of dried English yeast suspended in 10 cc. of H_2O + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 .

Flask No. 2. 0.2 g. of dried English yeast suspended in 10 cc. of water + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 .

Flask No. 1 showed the first signs of a colouration only after 4 minutes and eventually assumed a violet colouration which, although not so intense as in the case of washed yeast, was well marked. Flask No. 2 showed no colouration after 15 minutes.

Several other substances have been found, the addition of which to washed dried Munich yeast (Schroder) inhibits the action of the peroxydase. These are beef broth (0.1 cc.), sodium lactate (0.5 cc. of 1 per cent.), peptone (1 cc. of 1 per cent.), alkalis and acids. Beef broth previously treated with H_2O_2 for 20 minutes still has an inhibiting effect. As to the acids and alkalis, small traces of either will inhibit the action of the peroxydase in yeast as shown by the following experiment.

Experiment 7. Flask No. 1. 10 cc. of washed dried Munich yeast (Schroder) suspension (0.1 g.) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 0.5 cc. N/10 KOH.

Flask No. 2. 10 cc. of washed dried suspension (0.1 g.) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 0.5 cc. N/10 H_2SO_4 .

Flask No. 3 contained 10 cc. of washed dried yeast (Schroder) suspension (0.1 g.) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 .

No. 3 showed a colouration after two minutes. No. 1 and No. 2 showed no colouration after 5 minutes.

As the washings of dried yeast are slightly acid to litmus the possibility that the acidity of the washings acted as the inhibiting agent on the peroxydase suggested itself, but it was experimentally proved that this was not the case.

Experiment 8. In this experiment the washings were carefully neutralised to litmus.

Flask No. 1. 10 cc. of washed dried Munich yeast suspension + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 3 cc. H_2O .

Flask No. 2. 10 cc. of washed dried Munich yeast suspension + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 1.5 cc. of neutralised washings + 1.5 cc. H_2O .

Flask No. 3. 10 cc. of washed dried Munich yeast suspension + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 3 cc. neutralised washings.

No. 1 showed a colouration after 2 minutes while No. 2 and No. 3 showed no colouration after 5 minutes.

Precisely the same result was obtained when the washings were made neutral to phenolphthalein.

Washed and unwashed Munich dried yeast, washed and unwashed English dried yeast, washed and unwashed fresh English brewery yeast give no reaction with p-phenylenediamine and with benzidine in absence of H_2O_2 , which proves the absence of an oxydase. Similarly the above-mentioned yeasts under the same conditions give no reaction for tyrosinase.

CATALASE.

In the experiments of Bach mentioned earlier in this paper this experimenter also observed that the amount of oxygen evolved by the catalase of the zymin was almost equal to the theoretical amount capable of being liberated from the hydrogen peroxide. In order to ascertain with precision whether the washing of dried Munich yeast influenced the catalase, experiments were carried out as follows: 0.1 g. of dried Munich yeast (Schroder) was introduced into a small Erlenmeyer flask connected with an azotometer filled with mercury over which the liberated oxygen could be collected and measured. 25 cc. of hydrogen peroxide were run in from a burette passing through the stopper of the flask, and the volume of the oxygen liberated and the time taken for the liberation of the gas noted. Then a second experiment was carried out in which 25 cc. of the hydrogen peroxide were treated in the same apparatus with excess of acidified KMnO_4 , and the volume of oxygen liberated again measured. The available oxygen of the hydrogen peroxide, i.e. half the amount evolved on treatment with KMnO_4 , was thus ascertained and compared with the volume evolved by the action of the catalase of the yeast. The washed dried Munich yeast was treated in a similar way.

Experiment 1. 0.1 g. of unwashed dried Munich yeast liberated from 25 cc. of H_2O_2 17.1 cc. of oxygen in 5 minutes.

Acidified KMnO_4 and 25 cc. of the same H_2O_2 liberated 33.6 cc. of oxygen.

Experiment 2. 0.1 g. of washed dried Munich yeast liberated from 25 cc. of another sample of H_2O_2 11 cc. of oxygen in 5 minutes.

Acidified KMnO_4 and 25 cc. of the same H_2O_2 gave 21.9 cc. of oxygen.

It is evident from these results that the catalase in the dried Munich yeast is capable of liberating the entire available oxygen of the H_2O_2 and that the washing of the yeast makes no difference either to the volume of the oxygen liberated or to the rate of evolution.

INVERTASE AND MALTASE.

Experiments were next made to ascertain whether by repeated washing at ordinary room temperature it was possible to remove all traces of invertase and maltase from dried Munich yeast (Schroder) and zymine. The investigations were carried out by means of two different sets of experiments. In one set the yeast was washed six times. The first washings were boiled in order to destroy any invertase and maltase they might contain and added to the washed yeast, the mixture being then digested with cane sugar and maltose respectively for 17 hours at 25° and the CO₂ evolved, if any, noted. One flask containing glucose and another containing water in addition to the mixture of washed yeast and boiled washings were also digested at the same time for comparison. In the event of the invertase and maltase being entirely washed out no fermentation of cane sugar or maltose could be expected to take place, whereas if hydrolysis occurred the resulting hexoses would be fermented by the zymase in presence of the coenzyme added in the washings.

In the second set of experiments the cane sugar and the maltose were respectively digested with the washed yeast without the addition of the washings for 3 hours at a temperature of 25°. The suspensions were then filtered and the filtrate examined in order to ascertain whether any hydrolysis had occurred. In this case, no coenzyme being present, no fermentation could take place.

1. INVERTASE.

Experiment 1. The Munich dried yeast used in this experiment was washed six times.

No. 1. 25 cc. (5 g.) of washed Munich yeast + 20 cc. (1 g.) of cane sugar solution + 30 cc. of boiled washings + 0.5 cc. of toluene.

No. 2. 25 cc. (5 g.) of washed Munich yeast + 20 cc. (1 g.) of glucose + 30 cc. of boiled washings + 0.5 cc. of toluene.

No. 3. 25 cc. (5 g.) of washed Munich yeast + 20 cc. H₂O + 30 cc. boiled washings + 0.5 cc. toluene.

The digestion was continued for 17 hours at 25°.

The contents of all three flasks fermented and the following volumes of CO₂ were evolved :

Time	Flask No. 1	Flask No. 2	Flask No. 3
After 30 minutes	8.9 cc.	8.2 cc.	3.6 cc.
„ 60 „	14.8	14.1	5.5
„ 90 „	18.8	17.7	7.2
„ 17 hours	54.8	57.3	16.0

An experiment in which zymin was substituted for the dried Munich yeast washed six times was also carried out and the results obtained were similar. In 17 hours 25.3 cc. of CO_2 were evolved from the cane sugar, 25.2 cc. from the glucose, and 4.9 cc. from the autofermentation of the zymin.

These results show that the rate of evolution and the volume of the CO_2 produced from cane sugar are almost the same as from glucose, and it may therefore be concluded that the amount of invertase left in the dried Munich yeast and the zymin after washing is very appreciable.

The above results are confirmed by the following set of experiments.

Experiment 2. The dried Munich yeast used was washed six times.

Flask No. 1. 25 cc. (5 g.) of washed dried Munich yeast + 20 cc. (1 g.) of cane sugar solution + 0.5 cc. of toluene.

Flask No. 2. 25 cc. (5 g.) of washed dried Munich yeast + 20 cc. of water + 0.5 cc. of toluene. The contents of the two flasks were incubated for 3 hours at 25° and were then filtered, and the ratio

$$\frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}}$$

of filtrate No. 1 was determined, the rotation of filtrate No. 2 being reckoned for in calculating the actual ratio.

$$\text{Ratio} \frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}} \text{ of filtrate No. 1} = \frac{-0.373}{0.500} = -0.746.$$

$$\text{Ratio} \frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}} \text{ of invert sugar} = -0.800.$$

Experiments similar to Experiment 2 were carried out with dried Munich yeast washed with a mixture of water and toluene and with zymin washed both with water only and with water and toluene. The following are the results obtained:

$$\text{With zymin washed with water} \quad \text{Ratio} = \frac{-0.586}{0.736} = -0.796.$$

$$\text{,, ,, ,, and toluene Ratio} = \frac{-0.629}{0.760} = -0.827.$$

With dried Munich yeast washed with toluene and water

$$\text{Ratio} = \frac{-0.614}{0.730} = -0.840.$$

All the above results show that the entire cane sugar was inverted during the three hours.

In view of these results it became of interest to ascertain whether there was any difference in the rate of inversion of cane sugar between washed and unwashed zymin. Zymin was chosen because small quantities of that preparation in a concentrated solution of sugar do not commence fermenting for

some time and consequently it is possible to ascertain the amount of sugar inverted during that time and compare it with the amount inverted in the same time by an equal weight of zymin after being washed.

Experiment 3. The mixtures were incubated for 20 minutes at a temperature of 25°.

Flask No. 1. 0.1 g. of unwashed zymin suspended in 20 cc. of water + 50 cc. of 10 per cent. cane sugar solution.

Flask No. 2. 0.1 g. of washed zymin suspended in 20 cc. of water + 50 cc. of 10 per cent. cane sugar solution.

No fermentation was observed during the twenty minutes. The flasks were then placed in boiling water for twenty minutes in order to stop any further action of the invertase. After cooling to the room temperature they were made up to 100 cc. and filtered and the reducing powers of the filtrates calculated in terms of glucose were determined.

Reduction of filtrate No. 1 = 1.04 g. of glucose per 100 cc.

„ „ No. 2 = 0.67 g. „ „ „

It is thus seen that about $\frac{1}{5}$ of the total cane sugar was inverted by the unwashed zymin during the twenty minutes and that 64.4 per cent. of the amount inverted by the unwashed zymin was inverted by the washed preparation. This would suggest that the amount of invertase left behind even after six washings is a very considerable fraction (approximately 65 per cent.) of that originally present.

2. MALTASE.

A series of experiments similar to those carried out with cane sugar were made with maltose.

Washed dried Munich yeast (Schroder) and washed zymin readily fermented maltose on addition of the boiled washings. The volumes of CO₂ were as follows:

	Maltose	Glucose	Autofermentation
Munich dried yeast	58.0 cc.	57.3 cc.	16.0 cc.
Zymin	25.1	25.2	4.9

As from cane sugar the rate of evolution and volume of the CO₂ evolved from maltose are almost equal to those given by glucose, and consequently the amount of maltase left behind in the preparations after washing must also be very considerable.

The resulting ratios $\frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}}$ of filtrates obtained after digesting maltose without the addition of the washings for 3 hours at 25° with dried Munich yeast (Schroder) and zymin respectively, each of these preparations washed with water alone and with a mixture of water and toluene, were :

Dried Munich yeast washed with water only $\frac{+ 1.095}{0.550} = + 1.99.$

„ „ „ „ and toluene $\frac{+ 1.848}{0.680} = + 2.7.$

Zymin washed with water only $\frac{+ 2.360}{0.574} = + 4.1.$

„ „ „ „ and toluene $\frac{+ 2.886}{0.490} = + 5.89.$

Ratio for glucose + 2.1. Ratio for maltose + 9.8.

It appears from these figures that the whole of the maltose was converted into glucose by the dried Munich yeast while only a part of the maltose was converted by the zymin. Washing with a mixture of water and toluene seems however to have made no great difference to either preparation.

The idea of the possibility of removing the maltase from the zymin by a more thorough process of washing suggested itself and an experiment was accordingly instituted in which the zymin was washed 8 times. Each time it was vigorously shaken up in the pot for several minutes and the last time it was placed in a shaker for an hour previous to being centrifuged. On addition of the boiled washings the mixture fermented maltose freely at a rate almost equal to that produced with glucose. It is therefore evident that the most thorough method of washing will not entirely remove the maltase from zymin at ordinary room temperature.

As in Experiment 3 with cane sugar, 50 cc. of 10 per cent. maltose were also digested with 0.1 g. of washed and unwashed zymin respectively for 20 minutes. The resulting filtrates obtained showed the following reductions per 100 cc. calculated in terms of glucose :

Washed zymin 2.2 per cent. Unwashed zymin 2.2 per cent.

In this case although only a part of the maltose was converted into glucose the amounts hydrolysed were equal in both cases, and therefore the quantity of maltase removed by the washing can only have been very small.

SUMMARY.

1. Fresh English yeast reacts with H_2O_2 and p-phenylenediamine for peroxydase.
2. Dried yeast (both English and Munich) does not react for peroxydase with p-phenylenediamine and H_2O_2 but on being washed it regains that activity.
3. The addition of the washings and some other reagents to washed dried yeast inhibits the action of peroxydase.
4. Washing does not affect the activity of the catalase of dried yeast (Schroder).
5. The power of hydrolysing cane sugar is partially but not entirely removed from zymoin and dried Munich yeast (Schroder) by washing at ordinary room temperature whilst the power of hydrolysing maltose is not affected.

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XXXVIII. THE CONDENSATION OF AROMATIC ALDEHYDES WITH PYRUVIC ACID.

By EVA LUBRZYNSKA AND IDA SMEDLEY (*Beit Memorial Research Fellow*).

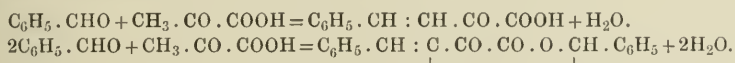
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In a preliminary communication published by one of us [Smedley, 1912], and in the foregoing paper [Smedley and Lubrzyńska, 1913], the condensation of certain fatty aldehydes with pyruvic acid in very dilute alkaline solution has been described.

In order to obtain a better knowledge of the reaction and hence possibly to overcome some difficulties with which we found ourselves confronted in our experiments in the aliphatic series we have investigated the behaviour of certain aromatic aldehydes under similar conditions. A number of the $\beta\gamma$ -unsaturated α -keto-acids have been prepared and converted by oxidation with hydrogen peroxide into the corresponding unsaturated acids containing one carbon atom less than the original keto-acid.

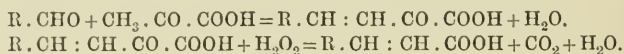
Claisen and Claparède [1882] studied the action of anhydrous hydrochloric acid on a mixture of benzaldehyde and pyruvic acid and obtained from the mixture cinnamoyl formic acid. This reaction was further studied by Erlenmeyer, junr. [1899, 1901], who showed that in addition to the above product γ -phenyl- β -benzylidene- α -ketobutyrolactone is formed:



The condensations of benzaldehyde, cinnamic aldehyde, piperonal and anisic aldehyde with pyruvic acid have been studied in dilute alkaline solution. The reactions proceed in all cases readily at the laboratory temperature. The amount of potash used was generally such that the strength of the solution was from 1/40 to 1/10 normal, and the mixture was allowed to stand at the temperature of the laboratory for periods varying from 2 to 7 days. As the reaction proceeds the aldehyde disappears and the

liquid gradually becomes yellow. When the reaction is completed, the keto-acid is thrown down on acidifying the liquid, and after purification, is oxidised to the corresponding $\alpha\beta$ -unsaturated acid.

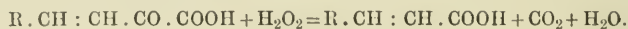
Yellow crystalline products are formed by the condensation of anisic aldehyde and piperonal respectively with pyruvic acid, the formulae of which are shown on analysis to agree with those required for the β -unsaturated α -keto-acids:



After we had completed the above series of experiments, we found that we had overlooked two papers by Erlenmeyer, junr. [1903, 1904], in which he had described the condensation of benzaldehyde and cinnamic aldehyde respectively with pyruvic acid in the presence of strong caustic soda (10 %). The products which we obtained on condensing cinnamic aldehyde with pyruvic acid were similar to those described by Erlenmeyer. He isolated two forms, an orange-red, melting at 75° and gradually converted *in vacuo* into a yellow form melting at 107°. Our products melted at 73° and 104° (uncorrected) respectively.

From anisic aldehyde and pyruvic acid yellow crystals melting at 130° were obtained. Piperonal under similar conditions gives beautiful yellow needles which at a temperature of about 70° undergo a remarkable transition into a deep orange-red form and finally melt at 163°. On keeping the red form so obtained in an evacuated desiccator or in a well-stoppered bottle it was still unchanged after a month had elapsed. If however it was exposed to the air it was gradually reconverted into the yellow variety.

On oxidation with the calculated quantity of hydrogen peroxide in neutral solution at the ordinary temperature the keto-acids split off carbon dioxide and form unsaturated acids:



Silver oxide which was successfully used to oxidise the corresponding keto-acids in the fatty series [Smedley, 1912], was found to be without action in the aromatic series.

EXPERIMENTAL.

Condensation of piperonal with pyruvic acid. 4 g. of pyruvic acid and 6 g. of piperonal were shaken up with 1 litre of water and 140 cc. N. KOH added; the mixture was allowed to stand at the ordinary temperature for a week, during which it became yellow. The small amount of unchanged

piperonal was filtered off and the solution neutralised with 36 cc. N. H_2SO_4 . The neutral solution was extracted with ether several times to free it from traces of unchanged aldehyde. Air was drawn through to remove the last traces of ether and the solution acidified. A yellow solid was precipitated and allowed to settle: it was then filtered off and well washed with cold water. After several recrystallisations from dilute alcohol it was obtained in beautiful yellow needles which turned to deep orange-red at about 70° and melted at 163° .

0.1062 g.; 0.2336 g. CO_2 ; 0.0368 g. H_2O .

C 59.98 %; H 3.85 %.

Calc. for $\text{C}_{11}\text{H}_5\text{O}_5$, C 60.00 %; H 3.63 %.

Condensation of anisic aldehyde with pyruvic acid. 5 g. anisic aldehyde and 3.5 g. pyruvic acid were shaken up with 1 litre of water and 70 cc. N. KOH added, the mixture being allowed to stand for 4 days during which a yellow colour developed. It was then neutralised with 25.05 cc. N. H_2SO_4 and the mixture treated exactly as in the case of the condensation of piperonal with pyruvic acid.

The yellow needles finally obtained melted at 130° and no change in colour was observed either on standing or on heating.

0.1100 g.; 0.2585 g. CO_2 ; 0.0500 g. H_2O .

C 64.09 %; H 5.00 %.

Calc. for $\text{C}_{11}\text{H}_{10}\text{O}_4$, C 64.07 %; H 4.85 %.

The specimen, part of which gave the above results on analysis, was allowed to remain in a closed test-tube for two weeks and then analysed:

0.0972 g.; 0.2330 g. CO_2 ; 0.0407 g. H_2O .

C 65.32 %; H 4.63 %.

A week later it was again analysed:

0.1208 g.; 0.2927 g. CO_2 ; 0.0562 g. H_2O .

C 65.98 %; H 5.16 %.

The acid was then recrystallised and again analysed:

0.1244 g.; 0.2972 g. CO_2 ; 0.0564 g. H_2O .

C 65.11 %; H 5.06 %.

The preparation was repeated and the freshly prepared product analysed:

0.1169 g.; 0.2743 g. CO_2 ; 0.0518 g. H_2O .

C 64.10 %; H 4.91 %.

The acid therefore on standing undergoes a slight decomposition, the nature of which was not ascertained.

Condensation of cinnamic aldehyde with pyruvic acid. 5 g. cinnamic aldehyde and 4 g. pyruvic acid were shaken up with 1 litre of water and 80 cc. of normal potash. The mixture was allowed to stand for 4 days at the

ordinary temperature, neutralised and treated as in the experiments already described with piperonal and anisic aldehyde.

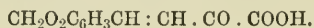
The condensation product was obtained in two forms; red crystals melting at 73° , which pass gradually into a yellow substance melting at 104° . In one experiment red crystals melting at 73° were exposed in an evacuated desiccator for 48 hours: they had then become yellow and melted at 95° . After another 24 hours the melting point was 101° and after recrystallisation from dilute alcohol it rose to 103° . Analysis showed that the composition of the two forms after drying in a vacuum desiccator was identical.

0.1274 g.; 0.3344 g. CO_2 ; 0.0600 g. H_2O .

C 71.58%; H 5.17%.

Calc. for $\text{C}_{12}\text{H}_{10}\text{O}_3$, C 71.28%; H 4.95%.

Oxidation of dioxymethylene-benzylidene-pyruvic acid:



The keto-acid was dissolved in alcohol, neutralised with potash and the theoretical quantity of hydrogen peroxide then added. It is important that no excess of the peroxide be present or the yield will be found to be diminished. The oxidation product was heated for an hour on a water-bath to complete the reaction and then acidified. A very pale yellow acid was precipitated, which after one recrystallisation was almost white and melted at 242° .

0.1074 g.; 0.2462 g. CO_2 ; 0.0423 g. H_2O .

C 62.47%; H 4.37%.

0.1148 g.; 0.2690 g. CO_2 ; 0.0458 g. H_2O .

C 62.51%; H 4.32%.

Calc. for $\text{C}_{10}\text{H}_8\text{O}_4$, C 62.50%; H 4.16%.

Oxidation of cinnamylidene-pyruvic acid:



The oxidation was carried out in a manner similar to that just described for the piperonal product. The acid obtained after recrystallisation from benzene and from alcohol melted at 165° and was therefore identical with the cinnamylidene-acetic acid prepared by Perkin from malonic acid and cinnamic aldehyde, melting at 165° .

0.0844 g.; 0.2335 g. CO_2 ; 0.0442 g. H_2O .

C 75.47%; H 5.80%.

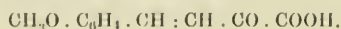
After again recrystallising:

0.1093 g.; 0.3052 g. CO_2 ; 0.0578 g. H_2O .

C 76.12%; H 5.85%.

Calc. for $\text{C}_{11}\text{H}_{10}\text{O}_2$, C 75.86%; H 5.74%.

Oxidation of methoxy-benzylidene-pyruvic acid:



This acid was dissolved in alcohol, carefully neutralised with potash and hydrogen peroxide added. In this case it was found that the best yield was obtained on adding 1.5 times the theoretical quantity of the peroxide. After several recrystallisations, the acid melted at 172° . Analysis gave the following result:

0.1020 g.; 0.2517 g. CO_2 ; 0.0530 g. H_2O .

C 67.23 %; H 5.70 %.

Calc. for $\text{C}_{10}\text{H}_{10}\text{O}_3$, 67.41 %; H 5.62 %.

Condensation of benzaldehyde with pyruvic acid. The condensation was carried out as in the cases already described, but the intermediate keto-acid was not isolated. The product was at once oxidised in neutral solution with hydrogen peroxide.

It was found in this case of the greatest importance to have the solution exactly neutral and to avoid any excess of the peroxide.

On acidification, cinnamic acid was precipitated. It was identified by taking the melting-point of a specimen mixed with pure Kahlbaum's cinnamic acid.

The yields in the above experiments varied from 50–70 % of the theoretical.

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LIX. THE ESTIMATION OF PYRUVIC ACID.

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(Received Nov. 11th, 1913.)

Pyruvic acid forms with phenylhydrazine a hydrazone by means of which, as Emil Fischer pointed out, one part of this acid in one thousand parts of water may be detected. The yield of hydrazone however is not quantitative and the attempts made by some authors to estimate pyruvic acid by weighing the phenylhydrazone precipitated have not proved satisfactory. Subsequently nitrophenylhydrazine was used as the precipitating reagent and by this means Neuberg and Karczag [1911] recovered 92% of pyruvic acid from a 1% solution, as the nitrophenylhydrazone. In estimating the amount of pyruvic acid in solutions containing 0.1% and of still lower concentrations this method is however of very little value, since the error introduced by the appreciable solubility of the hydrazone becomes of increasing importance with increasing dilution. An investigation into the action of the tissues on dilute solutions of pyruvic acid which I had undertaken had temporarily to be abandoned since the apparent removal of the acid observed might have been explained by an increase in the amount of hydrazone held in solution, and even when controls of corresponding dilution were used the results were unsatisfactory.

The action of asymmetrical diphenylhydrazine was investigated and was found to present similar difficulties.

If the experimental errors found above were due to the solubility of the hydrazone, the determination of the amount of phenylhydrazine removed from the solution in combination as hydrazone should give satisfactory results.

Estimation of Phenylhydrazine.

Fischer [1878] showed that phenylhydrazine was oxidised by cold dilute Fehling's solution with evolution of nitrogen; benzene and aniline were

formed and cuprous oxide precipitated. Strache and Kitt [1892] estimated the volume of nitrogen liberated and showed that if boiling solutions were used, no aniline was formed and the whole of the nitrogen was liberated in the free state; under these conditions six molecules of cupric oxide were necessary to oxidise two molecules of phenylhydrazine, a mixture of benzene and phenol being obtained. The reaction may be represented as follows:



The benzene formed during the reaction exerts an appreciable influence on the vapour tension, a difficulty which Strache overcame by saturating the gas both with benzene and with water vapour and introducing the necessary corrections. Strache [1891, 1892] estimated ketones and aldehydes by allowing warm solutions of the carbonyl compound and phenylhydrazine to react and then measuring the excess of phenylhydrazine in the solution by determining the volume of nitrogen evolved when oxidised by boiling Fehling's solution.

The method is not very convenient and it would be preferable to estimate the cuprous oxide formed. By the above method, however, in working with tissue-extracts containing pyruvic acid, any sugar present would react with the boiling Fehling's solution. If, however, the cuprous oxide formed when the excess of phenylhydrazine reacts with Fehling's solution at air temperature be estimated, this difficulty can be obviated.

Experiments were therefore made in order to determine whether the amount of cuprous oxide precipitated by a certain weight of phenylhydrazine was constant.

The Fehling's solution was made up as in Bertrand's method for estimating glucose.

Solution I.	Copper Sulphate crystals 40 grams per litre.
Solution II.	Rochelle Salt 200 grams } per litre.
	Caustic Soda 150 " }

5 cc. of a solution of phenylhydrazine containing 3.5236 g. in 100 cc. of 50% acetic acid were diluted to 100 cc. with water, and allowed to stand at the ordinary temperature for 30 minutes: in nine experiments, 20 cc. of each of the Fehling's solutions, I and II made up as above, were added to 10 cc. of the diluted phenylhydrazine solution and the mixture allowed to stand at the ordinary temperature for times varying from half an hour to four hours and a half. The cuprous oxide formed was then filtered through a Gooch crucible, dissolved in ferric sulphate solution as in Bertrand's method for the estimation of glucose and the ferrous sulphate produced titrated with deci-normal permanganate solution.

Results.

Time	Cc. N/10 KMnO ₄ equivalent to Cu ₂ O formed
30 minutes	6.0
60 "	6.05
90 "	6.0
150 "	6.05
150 "	6.0
180 "	6.0
210 "	6.0
240 "	5.95
270 "	5.95

The reaction between phenylhydrazine and Fehling's solution appears therefore to reach a definite stage of equilibrium within half an hour at the ordinary temperature, after which no further oxidation proceeds.

In three experiments, the following values were obtained:

1 cc. of N/10 KMnO ₄ was equivalent to (1) 0.002962 gr. phenylhydrazine,
(2) 0.002962 "
(3) 0.002935 "

As the mean of these experiments therefore

1 cc. N/10 KMnO₄ is equivalent to 0.00295 g. C₆H₅NH.NH₂.

It may be mentioned that in filtering the cuprous oxide from the cold Fehling's solution, it is advisable to filter only under a slight difference of pressure, as there is a tendency for the cuprous oxide to pass through the asbestos. If this happens the filtrate should be filtered through a clean Gooch crucible and the two results added together.

Estimation of Pyruvic Acid.

A specimen of Kahlbaum's pyruvic acid was distilled under diminished pressure and the fraction boiling at 77°–78° under a pressure of 15–20 mm. used for the estimation. The method adopted was as follows:

A solution of pyruvic acid was made up containing 1.5228 g. per 100 cc. Quantities of from 2 to 10 cc. of this solution were diluted to about 80 cc., 5 cc. of a solution of phenylhydrazine, approximately 4%, added, and the mixture made up to 100 cc. and allowed to stand half an hour at the ordinary temperature; 5 cc. of the hydrazine solution were diluted to 100 cc. and allowed to stand for the same time. After half an hour the pyruvic hydrazone which had separated was filtered off and 10 cc. of each filtrate

5 cc. of a solution of phenylhydrazine acetate, 10 cc. of a solution of pyruvic acid and 10 cc. of a 1% glucose solution were made up to 100 cc. and the pyruvic acid estimated as above, and compared with a solution similarly made up but from which the glucose was omitted.

Residual hydrazine (without glucose)	required	4.80 cc. N/10 KMnO ₄ .
„ „ (with „) „	4.85 „ „	

The method therefore gives satisfactory results in estimating solutions of concentrations above 0.03%. Below this concentration probably more accurate results would be obtained by using a more dilute solution of permanganate.

The advantages may be summarised as follows:

- (1) It is easily carried out; the whole estimation can be done in little more than an hour.
- (2) It gives a greater degree of accuracy than the unsatisfactory method of gravimetric estimation at present in use.
- (3) The presence of glucose does not interfere with the estimation.
- (4) The method promises to be of general value for the estimation of carbonyl compounds and also for measuring the rate of interaction of these compounds with phenylhydrazine.

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[From the *Proceedings of the Physiological Society*, February 14, 1914.]
Journal Physiology, Vol. XLVIII.

A simple and convenient form of bicycle ergometer. By
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The ergometer is of the brake-type. The work done in foot pounds is calculated from the frictional pull, the revolutions and the circumference of the wheel. Although this type was formerly used by physiologists, it has been abandoned of late years in favour of more complicated devices, such as driving a dynamo from the bicycle and measuring the voltage and current developed (Atwater), or substituting a copper disc for the hind wheel, which is run between the poles of an electro magnet, thus constituting an electric brake (Krogh, Benedict and Carpenter). The present form was devised for experiments in hot or moist atmospheres extending over considerable periods and the principal ends aimed at were moderate accuracy and independence of any assistance, that is, the experimenter working the bicycle to be able to make all the necessary observations.

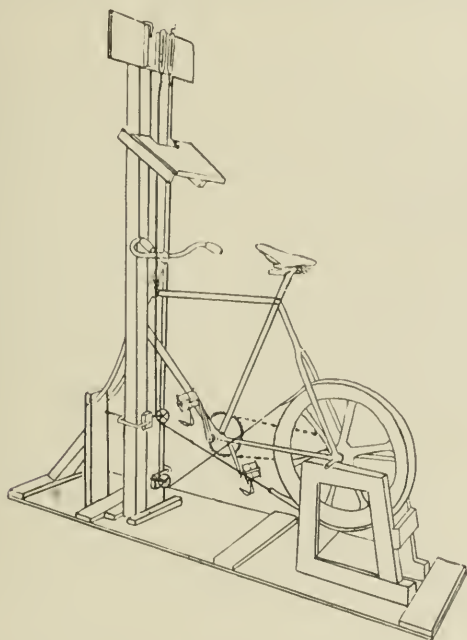
By gradual improvements these objects were more than attained and the instrument now has the following properties:

1. It is simple and comparatively inexpensive.
2. It has an error of less than 1 %.
3. The frictional pull is steady and is nearly independent of the velocity, so that it is not necessary to always pedal at a constant rate¹.
4. The instrument can be adjusted for any rate of work at any pedal-revolution desired.
5. The friction-band is quite thin and so does not get hot and vary the pull during the experiment.
6. Should for any reason, *e.g.* the hydrometric state of the atmosphere, the tension of the cords vary, this is at once seen, the balances being in front of the eyes, and can be corrected by a fine screw movement.

The general design of the apparatus will be seen from the accompanying woodcut. It consists of a bicycle frame supported by a wooden stand, from the front of which two uprights ascend and carry a small desk, and a cross piece which provides for the attachment directly in front of the worker of the balances and other pieces of apparatus which may be necessary. A cast-iron wheel, $5\frac{1}{2}$ feet in circumference, weighing 22 kilos. and mounted on ball bearings, is substituted for the

¹ A frictional pull of 5 lbs. increased by half an ounce when the velocity of pedalling varied from 20 to 200 per minute *i.e.* 0.6 %.

back wheel. This wheel is one inch wide at the periphery, has slightly bevelled edges and is absolutely true. To the circumference of the wheel a thin band of linen or stout calico (such as a fracture bandage) with hemmed edges is applied and the two ends of the band are attached by whipcord to spring balances, having a range of 6 lbs. and graduated to one ounce, attached to a board on the top of the uprights. Hemming the edges of the bandage makes them slightly shorter than the centre and ensures the belt keeping in position on the fly-wheel. The cords to the balances pass round ball-bearing aluminium pulleys¹ in order to give them the right direction. These pulleys are mounted on blocks of wood which are attached to the lower part of the uprights by clamps.



In using the instrument the tension of the cord is roughly adjusted by moving the pulleys up or down, so as to provide the friction required; the final adjustment is made by turning the screw of a slide rest to which one of the balances is attached. The revolutions of the wheel are recorded by a cyclometer.

¹ Such as are used for making simple Atwood machines for schools, cost, 3s. 6d.

THE BLOOD VOLUME IN ANKYLOSTOMIASIS.

WITH SOME BIOLOGICAL NOTES RELATING TO THE DISEASE.

BY WILLIAM NICOLL, M.A., D.Sc., M.D., D.P.H.,

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Introductory.

THE investigations which form the subject of the present paper were carried out during my tenure of the Ernest Hart Memorial Scholarship in State Medicine of the British Medical Association. The experimental part of the work was conducted at the Lister Institute of Preventive Medicine, London.

The suggestion of the line of research came from Dr J. S. Haldane, F.R.S., who referred me to certain results obtained by Boycott and himself (1903). The essential question to be decided was the nature of the changes in the blood volume in Ankylostomiasis or hook-worm anaemia. In the paper referred to Boycott and Haldane, using the carbon monoxide method of estimation, found that in three patients suffering from hook-worm anaemia the blood volume was increased to a very considerable extent, while the total haemoglobin remained practically unchanged. The important bearing of this result upon the nature of anaemia in general appeared to demand confirmatory evidence and it was thought that such might be readily obtained by means of animal experimentation.

In setting out to deal with such a problem experimentally, several questions naturally suggested themselves. These were, in the first place, the exact nature of the disease as it is exhibited in man, its etiology, pathology and clinical identity; in the second place, the nature of the disease in the animals to be experimented upon and the question as to whether it is identical with, analogous to, or different

from the disease in man. In addition there was the question as to the possibility of accurately determining normal conditions in the experimental animals and finally various questions relating to idiosyncrasy, susceptibility and immunity.

With regard to the nature of the disease in man it is universally accepted that the prime etiological factor is the presence in the intestine of either or both of two species of parasitic worms, namely *Agchylostoma¹ duodenale* and *Necator americanus*. As to the direct or immediate cause of the anaemia, however, opinion is yet to a large extent empirical. From the habits of the worms of fixing on to and damaging the intestinal mucosa, and so leading to haemorrhage, it might at first sight be concluded that the causation of the anaemia could be fully explained by the resulting loss of blood. The anaemia would accordingly be of the secondary or haemorrhagic type. Against this, however, must be put certain facts of considerable weight. In the first place Looss (1911) insists that the worms do not suck blood and that their natural food is the intestinal epithelium. This view, in the latter respect, is supported by several observers, and by personal experience, to the effect that although blood is not infrequently found in the intestine of the worms the red blood corpuscles are not digested to any extent. In the second place the majority of observations point to the fact that intestinal haemorrhage is rare in cases of some duration, and thirdly the results of Boycott and Haldane give evidence that the blood content differs from that in secondary anaemias. These considerations, together with some of a more general nature, have led to the origin of the toxic theory. This in turn has of late developed along two distinct lines. On the one hand, it was believed that the worm itself secreted a toxic substance, the action of which gives rise to the anaemia; on the other hand, there is the more recent idea that the toxin is produced by some extraneous organisms such as intestinal bacteria, the products from which find their way through the damaged mucosa. Neither of these explanations, however, is entirely satisfactory. A number of attempts have been made to isolate the toxic substance and success has been reported by, amongst others, Preti (1908), Noc (1908) and Whipple (1909). The last mentioned, however, while confirming the presence of a haemolytic substance, maintains that it is too weak or present in too small quantities to be effective. Moreover the evidence that haemolysis does actually take place within the human body is conflicting and the latest observations by Ryffel are against the occurrence of any such

¹ This spelling is adopted in accordance with current zoological nomenclature.

process. Loeb and Smith (1904 and 1906), again, entirely failed to detect the presence of a haemolysin in the head of the worm, but at the same time they reported the presence of an anti-coagulating substance, which might possibly tend to favour the continuance of haemorrhage. The presence of this substance was confirmed by Noc (1908), but Liefmann (1905) could detect its presence only in a minority of cases.

The toxic theory of extraneous origin has of late been elaborated by Weinberg and Leger (1908). Their opinion is that the gravest forms of hook-worm anaemia can only be explained on the basis of a secondary microbic infection and they advance experimental proof in support of their belief. Whipple (1909), also, in discarding the haemolytic theory, comes to the conclusion that the anaemia is probably due to direct loss of blood accompanied by absorption from secondary foci of inflammation in the intestinal wall. Siccardi (1910) in a summarizing article concludes that the anaemia is chiefly of toxic origin but that the intestinal lesions and the haemorrhages are additional factors. Evidence from a different point of view is brought forward by Castellani (1910), who deals with the comparative frequency of fever in hook-worm infection in Ceylon. This fever he ascribes to the action of various intestinal, but not necessarily pathogenic, bacteria, amongst which a new form, *Bacillus asiaticus*, is particularly mentioned. The greatest objection, perhaps, to be raised against this secondary microbic theory is the rapidity with which the anaemia disappears on expulsion of the worms.

In dealing with the clinical aspects of the disease, one important point has to be borne in mind, namely, the distinction between hook-worm infection and hook-worm disease. Infection with the hook-worm, even in some cases to a very high degree, does not necessarily lead to disease. This fact has been observed by most workers who have studied the disease on a large scale and it is merely an instance of a general biological phenomenon, which is not capable of very ready explanation. Clinically the leading feature of hook-worm anaemia in man is the chronic and progressive nature of the disease, but although essentially chronic, the progress of the disease may in some cases be very rapid. The symptoms are those of severe anaemia, generally associated with some degree of gastro-enteritis. The large number of individual symptoms which may occur are not specifically characteristic of hook-worm anaemia. In the pallor and oedema, however, there is a greater resemblance to chlorosis than to pernicious anaemia. The blood shows

a more characteristic picture. The volume, according to Boycott and Haldane, may be increased by nearly 100 %, while the total oxygen capacity is only slightly decreased. The number of erythrocytes may fall as low as 1,000,000 per c. mm. while the haemoglobin percentage may be as small as 15 % or even 10 %. The colour index is generally considerably below unity. It is apparent that these facts give evidence of a close relation to secondary anaemia, the one essential difference being the fact that the total oxygen capacity is not materially diminished.

With regard to the blood cells several observers have remarked on the frequent occurrence of normoblasts and, in some cases, of megaloblasts. Boycott (1911), however, categorically denies the occurrence of such cells and argues, from their absence, that there is little regeneration of blood, "presumably because the blood contains as much haemoglobin as, and more red cells than, normal." In regard to the leucocytes the most important feature is the occurrence of eosinophilia. This was first noted in hook-worm anaemia (1891) by Mueller and Rieder, but since then it has been a matter of frequent remark, and it is now fairly well established that a high degree of eosinophilia frequently accompanies not only hook-worm anaemia but also hook-worm infection. At the same time it must be noted that the relation is not constant and that certain cases may show no eosinophilia even though heavily infected. This was pointed out by Low in the discussion on Boycott's paper in 1905 in the particular case of the natives of Uganda. It was found by Boycott (1911) that eosinophilia first became marked about two to three weeks after the initial infection and that it reached a higher degree about the time ova first appeared in the faeces, after which it tends to fall off but usually persists not only throughout the whole course of disease but even for many years after complete recovery.

Apart from bronchial asthma and scarlet fever, there are two classes of infection with which eosinophilia is particularly associated, namely parasitic worms and skin diseases. There is at present no satisfactory explanation of this remarkable association but the current theory is that the increase in the eosinophil cells betokens a reaction on the part of the marrow to toxins circulating in the blood. Such a theory is supported in the case of parasitic worms by the fact that encysted forms are frequently surrounded by a zone of cells, a large proportion of which are eosinophilic. Whatever the explanation, however, there can be no doubt that the occurrence of eosinophilia is not infrequently a useful

aid to diagnosis and it has indeed been employed by Boycott as a routine method in the case of miner's anaemia, the final and confirmatory diagnosis being made on examination of the faeces.

The disease in dogs and other animals.

With these facts in the case of the disease in man it is necessary to compare what is known in regard to similar conditions affecting animals, and here, as might be expected, the available facts are neither so numerous nor so carefully sifted. It is in the first place necessary to consider the class of worms to which the human hook-worms belong, for it has frequently happened that much confusion has been introduced from neglect of accurately determining the specific characters of the infective agent. In this wise epidemiological misconceptions have from time to time been promulgated, such, for instance, as the belief that dogs and other animals serve as carriers of the human hook-worms, or that the hook-worm passes through a sexual stage outside the body. *Agchylostoma duodenale* and *Necator americanus* may be characterised as bursate or Strongylid Nematodes, belonging to the family Agchylostomidae, the chief features of which, separating them from other bursate Nematodes, are the dorsal bending of the head, the presence of a large buccal capsule which is armed with symmetrical groups of teeth or cutting plates, but which lacks coronae radiatae. These characters are sufficient to differentiate the hook-worms from the Sclerostomes, on the one hand, and, on the other, from the "wire-worms" and lung worms of sheep and other Herbivora. It may be remarked that a considerable proportion of all those worms are capable of provoking haemorrhage and giving rise to anaemia and malnutrition. The Agchylostomidae, however, are regarded as particularly deserving of the title "blood suckers," although, as has already been pointed out, the term is possibly a misnomer. They are divided into two subgroups, *Agchylostominae* and *Bunostominae*, the former being parasites of Carnivora, the latter of Herbivora. The chief structural differences between these groups consist in the facts that the *Bunostominae* have an extra pair of internal teeth and an unpaired internal dorsal prominence (the so-called unpaired dorsal tooth of *Necator*) in the buccal capsule. *Agchylostoma* belongs to the first group, *Necator* to the second. As mentioned above it has from time to time been stated that *A. duodenale* occurs in the dog and other animals but it is now fairly conclusively established that this species is a specific parasite of man. The dog, the cat and the fox,

however, and probably some other Carnivores harbour a very similar species, *A. caninum*. Four additional species have been recorded from other Carnivores. Another genus, *Uncinaria*, contains a second parasite of the dog and cat, namely, *U. criniformis* and one or two other species occur in other Carnivores. *Necator americanus* is nearly as specific as *A. duodenale*, but it has been found, with certainty, in the gorilla. A second species, *N. africanus*, has been met with in the chimpanzee. The second genus of this group, namely *Bunostomum*, includes three species parasitic in cattle and sheep. Five other genera, comprising forms parasitic in various Herbivora, belong to this group. These include all the forms on which we have at present satisfactory information, but there are several others still imperfectly known.

That most of these parasites give rise to some form of anaemia is vouched for by several observers. *Bunostomum phlebotomum*, for instance, is credited (Ransom 1911) with being the cause of "salt-sickness" of cattle in Florida, which is characterised by, amongst a variety of other symptoms, progressive emaciation and pronounced anaemia, which in many cases terminates fatally. Like opinions in regard to the other species are not wanting, but it is chiefly with the disease as manifested in dogs that we are at present concerned. A form of pernicious anaemia in dogs, more particularly hunting dogs, has been known for a long time and its connection with hook-worm infection was first mooted in 1882 by Mégnin whose opinion was supported by Railliet and by Trasbot (1882), and was confirmed by further observations on the part of Mégnin (1883). From these we gather that the symptoms are as a rule much more severe than those in human hook-worm anaemia, and that the disease is on the whole less chronic and more fulminant in type. There is a similar debility, pallor, oedema and enteritis but there appears to be a greater tendency to vicarious haemorrhage especially in the form of epistaxis, and, to this, skin affections are added. That all the symptoms mentioned by these early authors constitute a single clinical entity must be a matter of some doubt. It is recognised that dogs are subject to anaemia of non-parasitic origin and it may be noted that in some of the cases of hook-worm anaemia only a few worms were found in the intestine. It is remarkable that the disease should only attack kennelled hunting dogs although it cannot be denied that the circumstances are especially favourable to continued and repeated infection. Later observations on this subject are scanty. Gray (1899) recorded the occurrence of ankylostomiasis in dogs in Assam and asserted that very profuse

haemorrhage was frequent. Powell recorded somewhat similar facts for Cochin China. Thiroux and Teppaz (1906) also record the occurrence of hook-worm disease in dogs in West Africa. It is not made clear, however, what proportion of the infected dogs the disease attacks or what the age-incidence or other epidemiological factors are. According to the observations and experimental work of Looss (1911) it would appear that only young dogs are liable to attack and Liefmann (1905) noted experimentally that older dogs resisted infection to a very considerable extent. A similar anaemic disease in cats was first observed in 1878 by Grassi.

With regard to the condition of the blood in the anaemia of dogs we have practically little or no information beyond the fact that the erythrocytes are diminished and the leucocytes and eosinophils increased.

From the foregoing remarks it is evident that idiosyncrasy and susceptibility play a certain, if little understood, part in the etiology of hook-worm infection and anaemia not only in man but also in dogs and probably other animals. In regard to hook-worm infection in man, age appears to have little importance, but in many animals and with other parasites besides hook-worms, only young animals are prone to infection. The same phenomenon is manifested in the case of the human thread-worm (*Oxyuris vermicularis*) which shows an overwhelming predilection for children and generally disappears after puberty. In the case of the human hook-worm however, idiosyncrasy is manifested in other directions in illustration of which may be quoted the fact that Boycott was apparently insusceptible to infection through the skin although he readily acquired infection through the mouth.

The blood-picture in normal dogs.

There is finally the consideration of the normal condition in the experimental animals. The blood-picture of dogs and cats has been dealt with by Paton, Gulland and Fowler (1902). They found that, normally, the dog has a wide range in the number of red blood cells, namely, from 5 to 9 millions per c.mm., the leucocytes from 11,000 to 26,000, and the haemoglobin index from 80 to 110. They also found an average of 3 to 4% of eosinophils. My own observations give figures agreeing in the main with these, namely, reds 5-8 millions, leucocytes 6-24 thousand, and Hb percentage 70-105, while the eosinophils vary from 0 to 4%. With regard to the normal blood volume several authorities have made observations which show a fair

amount of uniformity but diverge in respect of the limits. Ranke arrived at an average of $1/15$ of the body-weight, Jolyet and Laffont gave as limits $1/12$ to $1/13$; Panum found $1/12$ – $1/15$ and Heidenhain $1/12$ – $1/18$. These latter figures are quoted from Dreyer and Ray (1910), the original references not being available. More recently Abderhalden and Schmid (1910) have employed an optical method of estimation, in which they injected dextrin and determined the rotatory power of the blood before and after injection. By this means they found the blood volume to be $1/8$ – $1/9$ (11.3 – 12.4%) of the body weight. Dreyer and Ray, however, maintain that the blood volume is a function not of the body weight but of the body surface (*i.e.* of the $2/3$ rd power of the body weight approximately). They express their results in the form $B.N. = B.W. \cdot \frac{2}{3}/k$, where k is a constant determined experimentally for each species. It is evident that considerable discrepancy exists between the results of Abderhalden and Schmid and those of other observers. My own determinations on normal dogs give evidence of a comparatively wide range, the figures being $1/16$ – $1/10$ (6 – 10%) of the body weight, and this apparently independent of age or size. It has been found by Boycott (1912) and also by Dreyer and Ray (1910) that the blood volume per kilo of body weight is on the whole highest in small, *i.e.* young animals, and that it tends to decrease, though not periodically, as the animal increases in size. Boycott also concludes that the total haemoglobin (*i.e.* total oxygen capacity) is highest in small animals, although the haemoglobin percentage tends to rise as the animal grows.

Source of culture employed.

In view of what has been stated above and as a matter of expediency it was decided to conduct the experimental part of this investigation upon dogs, and, if possible, cats. Hook-worm disease in dogs is not known to occur naturally in this country and on that account some initial difficulty was experienced in obtaining the material for infection. This I eventually owed to the kindness of Prof. C. W. Stiles of Washington, U.S.A. A sample of egg-laden faeces from an infected dog was sent, and numerous eggs survived the journey of nearly a fortnight. From these a successful culture of infective larvae was obtained.

Method of estimation of blood volume.

Before proceeding to the determination of the blood volume in the infected dogs, preliminary control estimations were performed on three normal cats and five normal dogs. The method employed was a modified Welcker process, the details of which are as follows. The animal was carefully weighed, narcotised with morphine and then chloroformed. The carotid artery on one side was exposed and a three-way cannula was inserted. To this was attached a length of rubber tubing leading from the bottle containing the washing out fluid, and another clamped rubber tube by which the blood could be drawn off. The washing out fluid consisted of a 0.4% solution of potassium oxalate in 0.75% solution of sodium chloride in distilled water, which was kept at a temperature of 37°C. An accurately measured quantity of blood was then drawn off (25 c.c.), and mixed with an equal quantity of oxalate solution. This served as a standard. A further quantity of blood was then drawn off into a vessel containing oxalate solution, and this was continued for 5–10 minutes or until the flow became slow. Oxalate solution was then driven into the circulation from a pressure bottle, which was placed about six feet above the operating table. The solution was allowed to flow for 5–10 minutes, when more blood was drawn off. This was repeated several times for about an hour, by which time the heart had ceased to beat and the fluid drawn off had become somewhat pale in colour. The carotid artery was then clamped and the chest opened. A cannula was inserted into the aorta and connected with the oxalate bottle; the right auricle was incised. Oxalate solution was then perfused through the circulation and the fluid returning from the right auricle was collected in the thoracic cavity, whence it was sucked by means of a water pump into a collecting bottle. All the while the muscles and liver were firmly massaged. The lungs were completely washed out by a similar procedure. This process was generally discontinued after about two hours, by which time the fluid washed out, although it had not entirely lost colour, had become extremely pale. All the washings were then mixed and the total accurately measured. About a litre of this was retained for estimation. Meanwhile the animal was skinned and the flesh removed from the bones, along with the liver, spleen, heart and kidneys. The flesh and the bones were mashed up separately and then pressed to expel the juice which was collected and measured.

The standard sample of blood which had first been drawn off was then diluted to .5% with distilled water to which a few drops of chloroform were added to ensure haemolysis. The washed out blood was also diluted and haemolysed, and a small quantity was passed through filter paper to remove suspended matter. By this means a clear solution was obtained without losing more than a trace of the colouring matter. The difficulty of obtaining a clear solution from the pressed muscle juice was met by saturating it with sodium chloride and filtering. Allowance was made in the calculations for the increase in volume resulting from the salt added.

Each of these solutions was compared with the standard. Three test tubes of equal calibre were employed for the estimation. Into two was placed a quantity of the standard sample and into the third a measured quantity, usually 10 c.c., of the diluted washings. To this was added distilled water from a graduated burette until the tint matched that of the standard. The amount of water added was noted and from this the strength of the solution was estimated. The total volume could then be calculated.

In some of the earlier estimations the washings were collected in two or three successive portions and each was estimated separately. It was found that the last portion contained so little haemoglobin that no material addition would have been made by continuing the washing process longer. In the case of the muscle juice the presence of muscle pigment rendered the solution brown so that it was impossible to compare it accurately with the standard solution. On that account the estimation was made by means of the spectroscope. As a standard of comparison the dilution was taken which was just sufficient to cause the *D* line of the haemoglobin spectrum to disappear. Several of the early readings were confirmed by Prof. C. J. Martin, to whom I am much indebted for help in this and other matters. Further controls of the accuracy of these readings were effected by comparing the results obtained by this with those of the ordinary colorimetric method when a comparatively strong solution of haemoglobin was used. The discrepancy was found to be of small amount.

The quantity of blood in the washings, muscle juice and bone juice having thus been obtained, the sum of these plus the 25 c.c. originally withdrawn gave the total blood volume. The details in the control experiments were:

Cats (normal).		Weight (grams)	Blood vol. (c.c.)	Vol./Wt. (%)	Percentage washed out
A.		4200	189.5	4.70	96.2
B.		1990	98.9	4.97	97.3
C.		2415	116.9	5.07	96.6
Average		—	—	4.91	96.7

Dogs (normal).		Weight (grams)	Blood vol. (c.c.)	Vol./Wt. (%)	Percentage washed out
Z.		—	1130.2	—	92.9
E.		5400	525.2	9.72	98.1
F.		11000	1120.4	10.19	92.5
X.		9100	545.6	6.00	91.6
Y.		9800	744.3	7.59	96.0
Average		—	—	8.87	94.2

The complete Welcker method is a tedious process and one which in the case of a large animal could with difficulty be completed in a day. On that account it was decided to take the amount of blood washed out as a constant percentage (94% of the total volume). In the remaining experiments therefore only the amount of blood washed out was estimated and the volume calculated therefrom.

Experimental Series.

I do not propose to enter here into the full details of each experiment, which would involve endless repetition and be of little interest. Only the outstanding data will be given, with explanatory notes wherever necessary.

A. (Stock.)

Apr. 18th, 1910. Infection started. Wt. 5.9 kg. Hb. 106%. Erythrocytes 6.38 millions.
 May 5th. *Agchylostoma* ova in faeces. Wt. 6.4 kg.
 June 7th. Infection stopped.
 July 19th. Wt. 5.8 kg. Hb. 76%.
 Oct. 7th. Hb. 96%. Red cells 5.99 millions. Eosinophils 0%.
 „ 24th. Hb. 84%. Red cells 7.15.
 Apr. 20th, 1912. Alive and well. Several ova in faeces.
 June 22nd. Killed. Hb. 96%. Red cells 5.56. Wt. 8.3 kg. Blood volume 632 c.c.
 = 7.62% of body-weight.

B.

Apr. 19th. Infection started.
 „ 24th. Dead. Pneumonia. No hook-worms in intestine.

C.

Apr. 20th. Infection started.
 May 5th. Dead. Pneumonia. Large number of immature hook-worms in ileum.
 Considerable amount of blood in intestine.

D. (Stock.)

May 11th.	Infection started. Wt. 9.1 kg. Hb. 90 %.	Red cells 5.63.
June 13th.	Ova in faeces. Infection stopped.	
July 1st.	Hb. 79 %.	
Oct. 7th.	Hb. 77 %.	Eosinophils 0 %.
Apr. 20th, 1912.	Alive and well. Moderate number of ova in faeces.	

G.

Feb. 28th, 1911.	Infection started.	
Mar. 4th.	Dog ill.	
„ 9th.	Very ill.	
„ 16th.	Dead. P.M. no obvious lesions. 12 immature hook-worms in intestine.	Not much blood.

H.

Mar. 2nd, 1911.	Infection started.	
„ 9th.	Very ill.	
„ 14th.	Dead. Pneumonia. 12 young hook-worms in intestine; also a few round-worms. Large amount of haemorrhage.	

I.

Apr. 5th, 1911.	Infection started. Wt. 13 kg. Hb. 92 %.	Red cells 6.35.
May 2nd.	Wt. 12.6. Hb. 68 %.	Eosinophils 3½ %.
„ 21st.	Wt. 12.2. Hb. 77 %.	Eosinophils 1 %.
July 10th.	Wt. 12.3. Hb. 79 %.	Eosinophils 3 %.
Sept. 1st.	Wt. 12.7. Hb. 98 %.	Red cells 6.95. Eosinophils 0 %.
	erythroblasts 650 c.mm.	
	Killed. Blood volume 905 c.c. = 7.13 % of body-weight. P.M.: viscera practically bloodless. 28 hook-worms in intestine. Very little blood in intestine, but large number of haemorrhagic areas of considerable size.	

J.

May 4th, 1911.	Infection started. Weight 8 kg. Hb. 88 %.	Eosinophils 12½ %.
	Already infected with <i>Ascaris</i> .	
„ 29th.	No ova in faeces. Wt. 9.2 kg. Hb. 88 %.	
June 6th.	Few ova in faeces.	
July 10th.	Wt. 9.4 kg. Hb. 59 %.	Eosinophils 2 %.
„ 12th.	Hb. 75 %.	Red cells 6.41. Eosinophils 5 %.
„ 15th.	Wt. 10 kg. Hb. 68 %.	Red cells 6.5. Eosinophils 5 %.
	Blood volume 729.3 c.c. = 7.29 % of body weight. P.M.: intestine contained a considerable amount of bloody mucus, with small clots of blood in places. About 170 specimens of hook-worm were met with from jejunum down to caecum, also 21 specimens of <i>Ascaris</i> in jejunum.	

K.

May 17th.	Infection started. Wt. 15.1 kg. Eosinophils 3 %.	
„ 18th.	Hb. 88 %.	Eosinophils 3 %.
„ 29th.	Wt. 14.7 kg. Hb. 81 %.	
June 11th.	Ova in faeces.	
July 10th.	Infection stopped.	
„ 14th.	Wt. 15.8 kg. Hb. 101 %.	Red cells 6.90. Numerous ova in faeces.

Aug. 7th.	Infection restarted.
„ 21st.	Wt. 14.5 kg. Hb. 97 % ₀ . Eosinophils 0 % ₀ . Red cells 8.5.
Sept. 7th.	Infection stopped again.
„ 29th.	Wt. 17.5 kg. Hb. 97 % ₀ . Red cells 8.7.
Oct. 20th.	Wt. 17.1 kg. Hb. 105 % ₀ . Eosinophils 0 % ₀ .
	Killed. Blood volume 1878.4 c.c. = 10.98 % ₀ of body wt. P.M.: some injection in mesentery. Large amount of bright red blood in rectum and patches throughout the intestine, with numerous haemorrhagic points. 146 specimens of hook-worm dispersed throughout whole length of intestine, 9 being found in the rectum and 3 in the caecum. All were adult and many were full of blood.

L.

May 18th, 1911.	Infection started. Wt. 6.6 kg. Hb. 95 % ₀ . Eosinophils 4 % ₀ .
June 11th.	Numerous ova in faeces.
„ 20th.	Weak. Wt. 5.6 kg. Hb. 53 % ₀ . Eosinophils 15½ % ₀ .
July 10th.	Hb. 55 % ₀ . Red cells 5.94. Eosinophils 6 % ₀ .
„ 11th.	Wt. 6.3 kg. Hb. 51 % ₀ . Red cells 5.98. Eosinophils 3 % ₀ .
Aug. 21st.	Wt. 7.9 kg. Hb. 99 % ₀ . Red cells 8.02. Eosinophils 0 % ₀ .
Sept. 7th.	Infection stopped.
Oct. 3rd.	Wt. 7.0 kg. Hb. 93 % ₀ . Red cells 5.83. Eosinophils 1 % ₀ .
„ 26th.	Wt. 7.1 kg. Killed.
	Blood volume 427.2 c.c. = 6.02 % ₀ of body weight. P.M.: intestine full of blood. 130 hook-worms, including several in caecum and rectum.

M.

May 22nd.	Infection started. Wt. 9.4 kg. Hb. 104 % ₀ . Eosinophils 3 % ₀ .
June 15th.	Ova in the faeces.
July 14th.	Wt. 8.9 kg. Hb. 81 % ₀ .
Sept. 7th.	Infection stopped.
Oct. 5th.	Wt. 8.1 kg. Hb. 95 % ₀ . Red cells 7.04. Eosinophils 0 % ₀ .
Dec. 5th.	Wt. 8.5 kg. Hb. 98 % ₀ . Killed.
	Blood volume 769.5 c.c. = 9.05 % ₀ of body weight. P.M.: very little blood or signs of haemorrhage. 30 hook-worms in intestine.

O.

Aug. 3rd.	Infection started. Wt. 8.3 kg.
„ 17th.	Wt. 7.6 kg. Hb. 86 % ₀ . Red cells 5.0. Eosinophils 0 % ₀ .
„ 24th.	Very ill. Infection stopped.
„ 30th.	Dead. Wt. 5.3 kg.
	P.M.: very much emaciated. Mesentery greatly injected. Lower part of intestine, caecum and rectum of dark green colour from diffused blood. Intestine contained a small amount of blood. Other organs normal. 318 hook-worms found in intestine. In trachea were found two living <i>Achylostoma</i> larvae (.62 mm. in length), one was found in oesophagus and five in stomach, measuring .66-.74 mm. in length; also one small immature female, measuring 3.1 mm., in the stomach.

P.

Aug. 9th.	Infection started. Wt. 5.5 kg.
„ 16th.	Hb. 68 % ₀ . Red cells 4.07. Eosinophils 1 % ₀ . Ill and weak.
„ 24th.	Very ill. Infection stopped. No ova in faeces.
„ 25th.	Dead. Wt. 4.4 kg.

P.M.: much emaciated. Mesentery greatly injected. Viscera very pale. Lower part of intestine and rectum of dark green colour and containing a large quantity of blood. In intestine were found 1886 hook-worms, 12 specimens of *Dipylidium caninum*, and one *Ascaris*. No larvae in lungs or trachea.

Q.

Sept. 26th.

Infection started. Wt. 7.8 kg.

Nov. 9th.

Killed. Wt. 7.8 kg. Hb. 108 $\frac{9}{10}$.

Blood volume 493.1 c.c. = 6.49 $\frac{9}{10}$ of body weight. P.M.: only two hook-worms found in intestine. No blood.

S.

Jan. 20th, 1912.

Wt. 3.0 kg.

Feb. 23rd.

Wt. 5.0 kg. Hb. 70 $\frac{9}{10}$. Eosinophils $\frac{1}{2}$ $\frac{9}{10}$.

Apr. 9th.

Wt. 9.0 kg.

May 3rd.

Wt. 6.5 kg. Hb. 86 $\frac{9}{10}$. Red cells 6.32. Whites 15.0. Eosinophils 4 $\frac{9}{10}$.

„ 7th.

Wt. 6.8 kg. Hb. 76 $\frac{9}{10}$. Red cells 5.09. Whites 12.3. Eosinophils 2 $\frac{9}{10}$.

„ 10th.

Wt. 6.9 kg. Hb. 80 $\frac{9}{10}$. Red cells 5.10. Whites 15.8.

„ 13th.

Wt. 7.1 kg. Hb. 82 $\frac{9}{10}$. Red cells 6.19. Eosinophils 1 $\frac{9}{10}$.

Infected with *Ankylostoma* larvae by the mouth.

„ 14th.

Wt. 7.7 kg.

„ 16th.

Wt. 7.6 kg. Hb. 72 $\frac{9}{10}$. Red cells 5.15. Whites 15.7. Eosinophils 0 $\frac{9}{10}$.

Erythroblasts 235.

„ 18th.

Wt. 7.4 kg. Hb. 72 $\frac{9}{10}$. Red cells 5.69.

„ 22nd.

Wt. 8.3 kg. Hb. 74 $\frac{9}{10}$. Red cells 6.50. Whites 20.1. Eosinophils 1 $\frac{9}{10}$.

Erythroblasts 502.

„ 25th.

Wt. 8.8 kg. Hb. 74 $\frac{9}{10}$. Red cells 5.79. Whites 11.8. Eosinophils 3 $\frac{9}{10}$.

Erythroblasts 1121.

„ 28th.

Wt. 8.9 kg. Hb. 76 $\frac{9}{10}$. Red cells 6.17. Whites 15.2. Eosinophils 6 $\frac{1}{2}$ $\frac{9}{10}$.

Erythroblasts 304.

„ 29th.

Wt. 9.0 kg. Hb. 72 $\frac{9}{10}$. Red cells 5.90. Eosinophils 10 $\frac{1}{2}$ $\frac{9}{10}$. Erythroblasts 1292.

June 1st.

Wt. 9.2 kg. Whites 18.4. Eosinophils 2 $\frac{9}{10}$. Erythroblasts 11,960.

„ 3rd.

Wt. 8.8 kg. Hb. 60 $\frac{9}{10}$. Red cells 4.89. Whites 9.5.

„ 6th.

Wt. 8.2 kg. Hb. 52 $\frac{9}{10}$. Red cells 3.32. Whites 14.0.

„ 7th.

Wt. 8.1 kg. Hb. 56 $\frac{9}{10}$. Red cells 3.57. Whites 18.8. Eosinophils 2 $\frac{1}{2}$ $\frac{9}{10}$. Erythroblasts 3102.

„ 9th.

Wt. 7.3 kg. Hb. 52 $\frac{9}{10}$. Red cells 4.05. Whites 18.7. Eosinophils 6 $\frac{1}{2}$ $\frac{9}{10}$. Erythroblasts 654. Temp. 98.8. Very ill. Thin and feeble.

No bleeding and apparently no pain or tenderness.

„ 10th.

Wt. 7.0 kg. Hb. 50 $\frac{9}{10}$. Red cells 3.77.

„ 11th.

Wt. 6.8 kg. Hb. 50 $\frac{9}{10}$. Red cells 3.29. Whites 22.7. Eosinophils 2 $\frac{9}{10}$. Erythroblasts 12,031.

„ 14th.

Wt. 6.5 kg. Hb. 48 $\frac{9}{10}$. Red cells 3.16. Whites 19.5. Eosinophils 4 $\frac{9}{10}$. Erythroblasts 1462.

„ 15th.

Wt. 6.8 kg. Hb. 48 $\frac{9}{10}$. Red cells 2.97.

„ 18th.

Wt. 6.6 kg. Hb. 40 $\frac{9}{10}$. Red cells 4.17. Whites 20.8.

Killed. Blood volume 490 c.c. and 7.43 $\frac{9}{10}$. 791 hook-worms in intestine, which contained considerable quantity of blood.

T.

May 7th, 1912.	Wt. 4.3 kg. Hb. 78 $\frac{0}{100}$. Red cells 5.97. Whites 7.7. Eosinophils 2 $\frac{0}{100}$. Erythroblasts 422. Temp. 102.2. Already infected with <i>Ascaris</i> .
„ 10th.	Wt. 4.4 kg. Eosinophils 0. Erythroblasts 38. Temp. 102.4° F.
„ 14th.	Wt. 4.4 kg. Hb. 78 $\frac{0}{100}$. Red cells 5.67. Whites 11.9. Eosinophils 1 $\frac{0}{100}$. Erythroblasts 0. Temp. 101.6° F.
„ 16th.	Wt. 4.2 kg. Hb. 82 $\frac{0}{100}$. Red cells 5.55. Whites 7.0. Eosinophils 0 $\frac{0}{100}$. Temp. 101.8.
„ 19th.	Wt. 4.5 kg. Hb. 88 $\frac{0}{100}$. Red cells 6.34. Whites 8.9. Eosinophils 4 $\frac{0}{100}$. Erythroblasts 177. Temp. 101.3–102.7° F.
„ 22nd.	Wt. 4.9 kg. Infection started. Fed by mouth.
„ 24th.	Wt. 4.9 kg. Hb. 78 $\frac{0}{100}$. Red cells 549. Eosinophils 0 $\frac{0}{100}$. Erythroblasts 1327.
„ 25th.	Wt. 5.0 kg. Hb. 70 $\frac{0}{100}$. Red cells 5.36. Whites 12.4. Eosinophils 7 $\frac{0}{100}$. Erythroblasts 3968. Temp. 102° F.
„ 29th.	Wt. 5.4 kg. Hb. 62 $\frac{0}{100}$. Red cells 4.42. Temp. 101.5° F. Eosinophils 3 $\frac{0}{100}$.
„ 31st.	Wt. 5.1 kg. Hb. 56 $\frac{0}{100}$. Red cells 3.98.
June 1st.	Wt. 5.0 kg. Hb. 52 $\frac{0}{100}$. Red cells 3.71. Temp. 101.8° F. Pulse 100.
„ 3rd.	Wt. 4.7 kg. Hb. 16 $\frac{0}{100}$. Red cells 90. Whites 19.7. Eosinophils 1 $\frac{0}{100}$. Erythroblasts 12,903. Temp. 98.8° F. Pulse 182. No ova in faeces. Very ill.
„ 4th.	Wt. 4.6 kg. Hb. 12 $\frac{0}{100}$. Red cells .88. Whites 20.7. Pulse 104. Temp. 94.4° F.
	Killed. Blood volume 293.7 c.c. = 6.38 $\frac{0}{100}$. 1719 hook-worms in intestine. Very large amount of blood in ileum, much of it bright red; also in caecum and large intestine. Several punched out pits in caecum. No larvae in trachea or oesophagus, but one found in stomach.

U.

Aug. 8, 1912.	Wt. 2.2 kg. Hb. 70 $\frac{0}{100}$. Red cells 5.48. Whites 21.1. Temp. 100.9° F. Already infected with <i>Ascaris</i> . Infected by mouth with hook-worm larvae.
„ 9.	Wt. 2.2 kg. Hb. 70 $\frac{0}{100}$. Red cells 5.75. Eosinophils 2 $\frac{1}{2}$ $\frac{0}{100}$. Erythroblasts 1793. No polychromasia. Temp. 101.3° F.
„ 10.	Wt. 2.2 kg. Whites 13.1. Eosinophils 2 $\frac{0}{100}$. Erythroblasts 1834. Temp. 101.8° F.
„ 12.	Wt. 2.3 kg. Hb. 75 $\frac{0}{100}$. Red cells 7.11. Temp. 100.8° F.
„ 13.	Wt. 2.5 kg. Whites 11.1. Eosinophils $\frac{1}{2}$ $\frac{0}{100}$. Erythroblasts 55.
„ 15.	Wt. 2.9 kg. Hb. 68 $\frac{0}{100}$. Red cells 6.56. Temp. 102.4° F.
„ 16.	Wt. 2.9 kg. Whites 18.4. Temp. 102° F.
„ 19.	Wt. 3.0 kg. Hb. 65 $\frac{0}{100}$. Red cells 7.72. Temp. 102.2° F.
„ 20.	Wt. 2.9 kg. Whites 15.8. Eosinophils 0 $\frac{0}{100}$. Erythroblasts 553. Temp. 101.6° F.
„ 21.	Wt. 3.1 kg. Whites 14.4. Eosinophils 0 $\frac{0}{100}$. Erythroblasts 720. Temp. 102° F.
„ 22.	Wt. 3.1 kg. Hb. 64 $\frac{0}{100}$. Red cells 8.35. Temp. 102° F.
„ 23.	Wt. 3.2 kg. Whites 11.4. Eosinophils 0 $\frac{0}{100}$. Temp. 101.4° F.
„ 29.	Wt. 3.6 kg. Hook-worm ova in faeces.
Oct. 15.	Wt. 5.8 kg. Still alive and well. No appearance of anaemia. Experiment discontinued.

V.

Aug. 8, 1912.	Wt. 2.0 kg. Hb. 70 $\frac{0}{10}$. Red cells 5.40. Whites 21.5. Eosinophils 3 $\frac{0}{10}$. Erythroblasts 430. Temp. 101.2° F. Already infected with <i>Ascaris</i> . Infected with hook-worm larvae through skin.
„ 10.	Wt. 2.0 kg. Hb. 68 $\frac{0}{10}$. Red cells 6.02. Temp. 102° F.
„ 12.	Wt. 2.0 kg. Whites 25.9. Eosinophils 3 $\frac{0}{10}$. Erythroblasts 388. Temp. 100.4° F.
„ 13.	Wt. 2.2 kg. Hb. 70 $\frac{0}{10}$. Red cells 6.86. Temp. 101.3° F.
„ 15.	Wt. 2.5 kg. Whites 15.6. Eosinophils 0 $\frac{0}{10}$. Temp. 102.2° F.
„ 16.	Wt. 2.5 kg. Hb. 66 $\frac{0}{10}$. Red cells 7.56. Temp. 102° F.
„ 19.	Wt. 2.6 kg. Whites 19.7. Eosinophils 0 $\frac{0}{10}$. Erythroblasts 197. Temp. 102.3° F.
„ 20.	Wt. 2.6 kg. Hb. 61 $\frac{0}{10}$. Red cells 6.99. Temp. 101.2° F.
„ 21.	Wt. 2.9 kg. Hb. 66 $\frac{0}{10}$. Red cells 6.43. Temp. 102.2° F.
„ 22.	Wt. 2.8 kg. Whites 14.7. Eosinophils 0 $\frac{0}{10}$. Erythroblasts 147. Temp. 101.3° F.
„ 23.	Wt. 2.9 kg. Hb. 65 $\frac{0}{10}$. Red cells 6.58. Temp. 102° F.
„ 29.	Wt. 3.0 kg. Hook-worm ova in faeces.
Oct. 15.	Wt. 4.8 kg. Still alive and well. No appearance of anaemia. Experiment discontinued.

*General remarks with regard to time of appearance
of ova in faeces etc.*

A dog which first received larvae on April 18th, 1910, showed numerous ova in its faeces on May 5th, *i.e.* 17 days afterwards. This period corresponds with that found by other observers *e.g.* Lambinet (1905), Looss (1911), who have experimented with the dog hook-worm. A second dog, infected on April 19th, died on April 24th from pneumonia. No worms or larvae were found in the intestine. A third dog, infected on April 25th, died on May 5th from the same cause. Over 100 specimens of *Agchylostoma caninum* were found in the ileum. They were adult but none of the females contained mature ova. There had been a considerable amount of haemorrhage in the intestine, bright red blood being found as far down as the caecum. The death of these two dogs was almost certainly not to be ascribed to the infection with *Agchylostoma* for two other dogs in the same batch died from pneumonia before infection was started. From the lungs of these dogs an organism of the *Pasteurella* group was isolated, but whether this was the cause of death or not was not determined.

The first dog escaped the pneumonic infection, and small quantities of larvae were administered to it regularly every second day for about a month. It remained alive and healthy for over two years, and continued to show a moderate number of ova in its faeces during the

whole of that period. From it, chiefly, the remaining dogs of the series have been infected. At no time did it display any very marked signs of anaemia, although the haemoglobin percentage was at one time as low as 76. Blood examinations were made only at irregular intervals on this dog. On one occasion, during an attack of mange, the Hb percentage was as high as 96. Later, however, on treatment and recovery, it fell to 84. The erythrocytes rarely fell below six million per c.mm., while the leucocytes varied from 14-25 thousand, the higher figures being recorded during the early stages of infection. At no time was there any degree of eosinophilia, eosinophils being frequently entirely absent.

A fourth dog was infected on May 11th. No ova could be found in the faeces up to the 20th day. They were first seen on June 13th (33 days), but were probably present some days earlier as examination was intermitted for about a week. The weight of this dog, which was full grown, remained practically constant at about nine kilograms. The haemoglobin percentage, which was at first 95-100, had fallen to 80 when infection was established, *i.e.* when ova were demonstrable in the faeces, and remained about that figure for a year. The red cells did not fall below five millions per c.mm., while the leucocytes were generally about 20,000. In this case, again, no evidence of eosinophilia was obtained, but in the faeces there were distinct signs of intestinal haemorrhage. This dog is still alive and well and its faeces still contain moderate numbers of hook-worm ova. It has been maintained as a reserve stock.

As already remarked no pronounced symptoms of anaemia were observed in these dogs even after a month's constant infection. Similar infection was prolonged for periods of three, six, and more months in the case of other dogs, but in no case could a severe chronic anaemia, similar to that occurring in man, be produced. These dogs were all reputably under one year old and some were believed to be not much over six months. With still younger dogs there was the constant difficulty of intercurrent affections, such as pneumonia and distemper, to which in the majority of cases they succumbed soon after infection was started. These difficulties delayed the progress of the experiments very considerably, and it was at last decided to examine the blood volume of such dogs as had been infected and had shown signs of anaemia even in a minor degree. The general course of infection in these dogs was the advent of a slight though distinct degree of anaemia about three to four weeks after infection was started, this continuing for

varying periods of a month or longer, and being followed by gradual recovery, and this in spite of continued infection. The Hb percentage in some cases sank as low as 50, but the red cells were rarely under 5,000,000. The Hb index was therefore usually decidedly under unity. In the young dogs which did not succumb to intercurrent affections the symptoms were much more severe and the disease rapidly terminated in death. It is to be regretted that the blood volume of these dogs was not estimated, but this, unfortunately, was deferred in the hope of obtaining a chronic affection, a hope, however, which was in no case realised.

The results of the blood volume estimations may be tabulated as follows:

	Weight (gms.)	Blood washed out (c.c.)	Total vol. (calculated)	Blood vol. body wt. (%)	Hb %	No. of worms	Length of infection (days)
A.	8300	595.0	632.0	7.62	—	12	796
I.	12700	851.2	905.0	7.13	96	28	150
J.	10000	685.6	729.3	7.29	68	170	72
K.	17100	1766.5	1878.4	10.98	105	146	129
L.	7100	401.7	427.2	6.02	95	130	134
M.	8500	723.4	769.5	9.05	98	30	170
N.	9800	714.7	744.3	7.59	—	2	264
Q.	7800	463.5	493.1	6.49	108	2	44
S.	6600	461.0	490.4	7.43	40	791	36
T.	4600	276.1	293.7	6.38	12	1719	13
Aver.	9230			7.60 (1/13)			

On comparing this table with that on page 379 it is evident that on the average the blood volume per kilog. is distinctly less in the infected dogs than in the normal, although at the same time it must be noted that the infected dogs were on the whole a somewhat heavier lot.

Calculating the blood volume according to the formula of Dreyer and Ray, and taking as the observed volume only the amount washed out, we obtain the following values of the constant "*k*."

Normal	Infected
E. 0.746	I. 0.638
F. 0.873	J. 0.672
X. 0.599	K. 0.386
Y. 0.477	L. 0.922
	M. 0.572
	N. 0.642
	Q. 0.836
	S. 0.763
	T. 0.927
Average 0.674	0.706

From this it would appear that if the blood volume is a function of the surface and not of the body weight there is on the average little difference between the infected dogs and the normal, but that if anything the volume is somewhat decreased; individually there are three very marked deviations, namely in the case of dogs *K* and *L* and *T*, the former of which has a decidedly increased volume and the latter a markedly diminished volume.

The oxygen capacity of the blood per kilo of body weight has also been calculated, in this case on the estimated total volume, and the figures are as follows:

Normal:—*E.* 14.6. *F.* 10.8. *X.* 19.0. *Y.* 16.7. Average 15.3.

Infected:—*I.* 12.1. *J.* 9.4. *K.* 21.9. *L.* 10.6. *M.* 16.4. *N.* 14.6. *Q.* 13.3.
S. 5.5. *T.* 2.4. Average 11.8.

From this we see that on the average the oxygen capacity per kilo of weight is diminished in the infected dogs, but that at the same time there is a considerable amount of variation even in the normal dogs. We notice the very high figure given by *K*, and the very low figures of *S* and *T*, in which there was massive infection and considerable bleeding into the intestine.

These results are so variable, even in the case of normal animals, that it is difficult to draw any very definite conclusions from them. One fact would appear, namely, that normal dogs show wide variation in the factors on which the results of these experiments are based and on that account it would be necessary to obtain some very wide deviations in order to arrive at any definite conclusion.

A further endeavour was made to deduce some relation between the change in blood volume and the number of worms present, and the length of infection, but as will be seen from the table above no constant relation is obvious.

There are two additional matters which must be discussed before attempting to draw any final conclusion. These are the absence of eosinophilia and the presence of erythroblasts. The diagnostic significance of eosinophilia has already been mentioned in the earlier part of this paper, and it is somewhat remarkable to find it so constantly absent in the animals under investigation. The only animals in which it was at any time noted were *J* and *L*. In the former it was present before infection was started, and from the fact that a large number of round worms were found in the intestine after death, it is not improbable that they had some connection with the eosinophilia. In the case of *L* the eosinophilia made its first appearance about a month after infection was

started, but it fell off considerably during the course of the succeeding month, and eventually disappeared. No ready explanation of this curious circumstance presents itself. It must serve, however, to emphasise the fact that eosinophilia is not by any means a constant or invariable accompaniment of infection with intestinal worms.

The marked occurrence of erythroblasts in some cases is also worthy of note. In the earlier of these observations no account was taken of these cells, and it is possible that they escaped notice. Latterly, however, they were looked for and their numbers estimated. They did not occur in every case, but were particularly noted in dogs *I* and *R*. In the former they were found in the very considerable number of 650 per c.mm. They were also noted, however, in the young uninfected pup *R* to the extent of 100 per c.mm. Later, after infection, the number rose to 300 and eventually to 1150 per c.mm., after which it again fell to 300. These cells were usually of the normoblastic type and no megaloblasts were seen. The majority had a central nucleus, an evidence of their recent formation, but all stages were seen up to those in which the nucleus was quite peripheral. No rosette-shaped or dividing nuclei were observed. To me it appears there can be little question that the presence of these cells gives evidence of a distinct and active regeneration of blood cells to meet, presumably, the loss by haemorrhage, and this lends support to the view that the intestinal haemorrhage plays some part in the causation of the anaemia, at any rate in the early part of the infection. The occurrence of erythroblasts in the young normal pup is not, I believe, an abnormal circumstance, as these cells are not infrequently observed in the blood of children and of young animals for a few months after birth.

Other features in regard to the leucocytes were noted during the course of these experiments, the most interesting of which was the occurrence of intense basophilia during attacks of mange.

In addition to these observations, others, of biological rather than clinical interest, may be mentioned. Some of these have already been remarked upon in the opening part of this paper. There is first of all the remarkable fact that while young dogs may be infected to an extent only limited by death, older dogs, on the other hand, appear to be susceptible only to a very restricted degree. This is a circumstance which led to very great delay in the present research. It was always found possible to infect dogs, irrespective of age, and in the course of two or three weeks ova were found in the faeces. It was a natural assumption that by continuing the administration of infective material,

a very gross infection would eventually supervene, but such, as has already been stated, did not prove to be the case. It is impossible to imagine what factor enabled them to withstand further infection and the problem is one which undoubtedly possesses considerable biological interest.

Of a similar nature were the facts derived from attempts to infect cats and monkeys with the dog hook-worm. The cat, as might be expected from zoological considerations, is to a certain extent susceptible to infection with parasites of the dog. Thus, we find the common tape-worm, *Dipylidium caninum*, as frequent in cats as in dogs. The round-worms ("*Ascaris canis*" and "*Ascaris mystax*") also appear, to a certain extent, to be common to both, although this is a matter for further and more accurate investigation. On the other hand, the tape-worms of the genus *Taenia* appear to be extremely specific. The cat has its own species, *T. crassicollis*, which is never found in the dog, and conversely, the numerous species of *Taenia*, met with in the dog, are never found in the cat. The hook-worms appear to occupy an intermediate position as regards specificity. One species, *Uncinaria criniformis*, has been recorded only from the dog, while *Agchylostoma caninum* has been recorded from both, although much more frequent in the dog.

I attempted a considerable number of infection experiments with kittens, about two to four months old, but the results were vitiated by the "distemper" which almost invariably followed. This "distemper" occurred at two well-marked periods, namely, about the third and sixth months. In a litter of half a dozen, half would succumb at the third month, the others recovering only to have a second attack at the sixth month, from which there would be a solitary survivor. The disease was usually of the broncho-pneumonic type but not infrequently accompanied by gastro-enteritis. In only three cases did the kittens live long enough to display hook-worm eggs in their faeces. Only one survived much beyond the sixth month and this animal continued to pass eggs for about six months, but at the end of nine months it had apparently got rid of infection for no eggs could be detected after this period. Of two adult cats, one became slightly infected, the other was absolutely insusceptible.

Similar experiments were tried with three monkeys (*Macacus*) but in no case did infection take place, although they were fed with infective material continuously for three months.

Finally the experiment was tried of personal infection, but this also

was unsuccessful, and although infective cultures have been handled both by myself and by my assistant very frequently during the last two years, without any stringent precaution, no infection has taken place in either. This fact lends support to the belief that the dog hook-worm cannot infect man, and disposes of the idea that the dog can act as the carrier of hook-worm infection in man, either by harbouring the human form or by spreading its own particular species.

Owing to my departure for Australia these experiments have had to be discontinued and some of the points which might have required further investigation have had to be left incomplete.

SUMMARY AND CONCLUSIONS.

In these experiments the hook-worm anaemia of dogs does not appear to be exactly analogous to the corresponding disease in man, but differs from it in two essential particulars, namely, that only young animals suffer and that in them its course progresses much more rapidly to a fatal termination.

Older dogs, although not altogether insusceptible, acquire infection only to a moderate extent, which gives rise to a minor degree of anaemia. From this they gradually recover, even in spite of repeated and continued attempts at re-infection.

The anaemia in young dogs was characterised by great loss of weight, emaciation, prostration and intestinal haemorrhage, but in no case was epistaxis observed.

The blood volume of dogs suffering from the minor degree of hook-worm anaemia is not materially altered, but if anything is somewhat diminished. The oxygen capacity of the blood per unit of body weight is also, on the average, somewhat decreased.

Infection is generally accompanied by distinct though not profuse haemorrhage, which is most marked in the early stages, but tends to disappear.

Eosinophilia was not a constant sign either of infection or of disease.

Evidence of blood regeneration was furnished by the appearance of large numbers of erythroblasts (normoblasts) which increased with the progress of the disease.

Cats are much less easily infected than dogs, and monkeys are altogether insusceptible. Man, also, were found to be insusceptible to infection with the dog hook-worm.

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XXXIII. A NOTE ON THE BASES OF GASWORKS COAL-TAR WHICH ARE BELIEVED TO BE THE PREDISPOSING CAUSE OF PITCH CANCER, WITH SPECIAL REFERENCE TO THEIR ACTION ON LYMPHOCYTES, TOGETHER WITH A METHOD FOR THEIR INACTIVATION.

PART I. AUXETIC ACTION.

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It has long been known that gasworks coal-tar, the pitch derived from it and soot (which contains tar), cause chronic dermatitis, warts and ulcers leading to epithelioma, in the skins of the workmen employed at the briquette (patent fuel) works and tar distilleries. This specific predisposition to cancer brought about by these commodities has recently formed the subject of Home Office enquiries. The clinical evidence shows that it is only what is commonly known as coal-tar and the pitch derived from it by distillation that have this action; tar and pitch obtained from the blast-furnaces are harmless. The mechanical irritation caused by both varieties is identical, so that it is evident that some chemical factor is responsible for the predisposition to cancer above referred to, a fact borne out by the incidence of pitch cancer and warts caused by liquid petroleum [Legge, 1911; Lush, 1911; Ross, H. C., 1913, 1].

Although it is well known that gasworks tar differs widely in its chemical composition from blast-furnace tar, the latter being manufactured at a much lower temperature, it must also be remembered that the tar made at the gasworks and coke-ovens which is dangerous, is derived mostly from bituminous coal; the harmless blast-furnace tar, on the other hand, being made from the harder Scotch coal. [Ross, 1913, 2.]

H. C. Ross and J. W. Cropper [1910] put forward a working hypothesis connecting the onset of carcinoma and the predisposition to it with chemical substances called auxetics and kinetics. Auxetics are substances (most of them are amino-bodies) which induce cell-division in white blood cells, such as lymphocytes and other cells; kinetics are another group of substances (including most of the alkaloids) which excite amoeboid movements in cells.

The method of testing for both auxetics and kinetics is to mix the suspected solution with agar jelly, and to spread some blood cells on the surface of films prepared from this jelly. If auxetics are present in sufficient strength the lymphocytes after 10 minutes incubation at 37° will be seen to exhibit characteristic division figures; if kinetics are present, the cells (being examined without incubation) show exaggerated amoeboid movements. It is this test that has been employed in the following chemical experiments; the full details of the method have been described by Ross and Cropper [1911, 1]. In every case the test was repeated several times and throughout the experiments gave consistent results.

The present paper is concerned only with auxetic action, and the clinical bearing of this is not discussed; the question of the isolation of kinetics from tar will be described at a later date. In investigating the question of pitch cancer, Ross and Cropper [1911, 2] have detected auxetics and kinetics in gasworks tar and pitch, in soot and in liquid petroleum they were however unable to detect any kinetic whatever, and only a trace of auxetic in the harmless blast-furnace tar and pitch. A long series of experiments was made by these investigators and the subject is also dealt with in the Report of the second Home Office enquiry on pitch cancer [Lush, 1913].

From observations made by Ross and Cropper it was found that on fractionally distilling tar which had been found capable of yielding an aqueous extract active in inducing cell-division, the fraction distilling between 260° – 320° comprised the whole of the active portion, that is to say the activity was associated with the anthracene fraction. Various pure substances known to occur in this fraction were tested by the above observers but found to be without action on lymphocytes. The following experiments were therefore undertaken to see whether the active agent or agents could be isolated.

EXPERIMENTAL.

Isolation of active basic fractions.

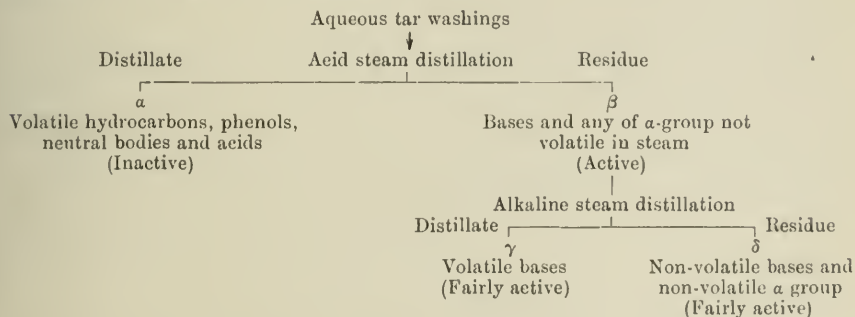
The tar used in the following experiments was a sample of the common variety which causes disease at the tar distilleries and briquette works and was kindly placed at our disposal by Mr Atkinson Butterfield.

Aqueous extracts were faintly acid in reaction.

Preliminary tests were made to ascertain the chemical nature of the substance or substances responsible for this auxetic action and in the first place an aqueous extract of the tar was used.

This was prepared by taking two volumes of water at 60° to 1 volume of tar, shaking for 1 hour, allowing to stand overnight and then centrifugalising or decanting off the aqueous layer. The washings prepared in this way were always faintly acid in reaction.

It was then found that on steam distilling with this original acid reaction, the substances capable of action on lymphocytes remained in the residue, but the kinetic action had apparently been destroyed or inhibited in some way as it was neither in the distillate nor residue. On steam distillation with a faintly alkaline reaction auxetic could be detected in both distillate and residue, that is to say the auxetic is slightly volatile in steam. The two steam distillations were then carried out consecutively on the same sample of washings and the result shown diagrammatically below, pointed to the active constituent being basic in character.



The active substance being therefore apparently of basic nature, acid extracts of the tar were made and it was found that this treatment yielded solutions which had a much stronger action on lymphocytes than had the aqueous ones. Acid extracts were accordingly used in all the following experiments, the acid employed consisting of two volumes of 5 per cent. hydrochloric acid to 1 of tar, and the extraction was carried out at 60° as in the previous case with water.

An attempt was then made actually to isolate the bases from the portion γ in the scheme above by means of chloroplatinic acid in the usual way, but after decomposing the platinum salt by means of sulphuretted hydrogen and then removing the excess of the latter by a current of air, no auxetic action could be detected. One explanation of this may be the adsorption of the active substance by the platinum sulphide which at first was formed in colloidal solution. A further explanation of this loss of activity was afterwards discovered and will be referred to later. The filtrate from the platinum salt was also found to be inactive after the removal of the platinum as above.

A further attempt was made using basic lead acetate as precipitant, decomposing the lead salt with sulphuric acid and then removing excess of acid by means of baryta. Here again no trace of auxetic action remained.

It then seemed necessary to obtain a larger and more concentrated supply of the active substance and methods with this end in view were next tried. It was found that on the addition of 10 per cent. caustic soda to an aqueous extract a precipitate was obtained which redissolved on continued addition of alkali and that this precipitate when filtered off and dissolved up in a little dilute acid (HCl) was a strong auxetic but did not keep well, rapidly losing its activity. In the same way the aqueous and acid extracts of tar likewise lose their activity on long standing in the air. With this method it was extremely troublesome to remove the excess of caustic soda, and if this is not done properly it is impossible to carry out the auxetic test with any hope of success as the alkali kills the cells, and an excess of salts even if neutral must be avoided¹.

The following method was then tried and found to be quite reliable. The tar was extracted as before with twice its volume of 5 per cent. hydrochloric acid, being shaken for one hour, and allowed to stand overnight and then centrifugalised. The supernatant liquor was precipitated with strong ammonia, care being taken not to add excess. A precipitate was obtained which on testing was found to be highly active while the supernatant liquor was inactive. The precipitate was collected, dried in a desiccator and again tested when dry and was still found to be highly active.

It is interesting to note that samples of alkaline condenser liquors and hydraulic main liquors from gasworks, which were kindly given by Dr Colman, were found after removal of sulphur to give the auxetic test. It is not certain whether these materials would be good sources of auxetics, as the latter deteriorate rapidly when exposed to the air in alkaline solution.

¹ The solutions must always be neutralised before testing on lymphocytes. Salts delay the diffusion of substances into cells so that they must not be in excess.

The precipitate from the ammonia may also be taken up in benzene and dried over anhydrous sodium sulphate, but before testing, the benzene must be very thoroughly blown off under a fan or distilled off under reduced pressure; the previous treatment is however to be preferred. In this way 85 g. of bases were extracted from about 1 cwt. of tar. These were distilled under reduced pressure and separated into five fractions.

Fraction	B.-P. at 16 mm.	Weight of distillate
I	0—100°	3.4 g.
II	100—150°	19.2
III	150—200°	18.2
IV	200—220°	8.5
V (Residue)	—	33

Fractions II and III were found to be highly active, I was extremely poor and IV and V were quite inactive.

II and III were then refractionated, but no great difference in activity could be detected in the fractions thus obtained. Both II and III oxidised rapidly in the air and in so doing lost their auxetic power, and this fact presumably accounts for the loss of action previously referred to in the weak aqueous and acid extracts of tar.

Further treatment of basic fractions.

Experiments were next made on the isolation of these active constituents and qualitative experiments showed the presence of nitrogen and possibly of sulphur.

An estimation kindly made by Dr Hartley of this Institute with van Slyke's apparatus showed that the nitrogen was not contained in any side chain but was bound up in the ring.

In this connection it was found that Kahlbaum's pseudocumidine, which is closely allied to the cumidine occurring in coal tar, was very feebly active. The test in this case requires great care as the conditions must be exactly right for any activity to be shown at all.

ψ -cumidine is easily benzoylated in the usual way yielding a white crystalline compound, Mp. 174–5° which has no longer any detectable action on lymphocytes. Attempts were then made to isolate individual bases from the mixture obtained by precipitation with ammonia, by means of benzoyl chloride, but little evidence of benzoylation could be obtained and no crystalline benzoyl compounds could be isolated. Had this been successful it would have been possible to separate the aniline bases from the pyridine bases and to ascertain whether auxetic action was an inherent property of either or both.

The next method tried was fractional crystallisation of the picrates. A saturated solution of picric acid was made, preferably in alcohol, and the basic fraction added drop by drop. A yellow solid at once separated out which crystallised readily from acetic acid. On fractional crystallisation two main fractions were obtained. The first more insoluble portion A, having a Mp. 199° – 201° , the second B (after several recrystallisations) Mp. 161° – 2° .

Analysis of A gave the following figures.

0.0895 g.; 0.1709 g. CO_2 ; 0.0219 g. H_2O .

C = 52.11 %, H = 2.71 %.

0.0999 g.; 0.1924 g. CO_2 ; 0.0252 g. H_2O .

C = 52.52 %, H = 2.80 %.

0.1188 g.; 16.8 cc. N_2 at 16° and 761.75 mm.

N = 16.52 %.

0.1319 g.; 19.1 cc. N_2 at 16° and 760.5 mm.

N = 16.80 %.

The figures obtained for picrate A correspond with the following empirical formula, $\text{C}_{22}\text{H}_{14}\text{O}_9\text{N}_6$ which requires

C = 52.17 % H = 2.76 % N = 16.60 %,

but nothing further is at present known with regard to the constitutional formula.

The quantity of picrate B, Mp. 161° – 2° , obtained was insufficient for analysis.

Experiments on the hydrolysis of picrate A were then undertaken with the object of isolating again and if possible identifying the active base from it, but the results so far have been somewhat unsatisfactory. In the first place the picrate was only hydrolysed with difficulty, and secondly it was found almost impossible to separate the very small amount of free base produced from the metallic picrates formed in the hydrolysis. Only when this was carried out with litharge was any free base obtained and the amount of this was much too small for identification to be possible.

The solution of this free base did however produce cell-division showing that it was one of the active constituents.

The hydrolyses were all carried out in air and possibly better results might be obtained by using an atmosphere of nitrogen, the active bases being very readily oxidised to inactive compounds.

Experiments on the inactivation of the auxetics of coal-tar.

In view of the theory that the susceptibility of pitch workers is due to the presence in the pitch used of the auxetics above described it seemed highly desirable to devise a method whereby these substances might be removed from the tar and pitch or at any rate inactivated. As has been

described above the auxetics can be almost completely removed from the tar by thorough washing with water or dilute acid. These methods however leave the tar in a state which renders its further distillation extremely troublesome and hence have practical objections.

A method has been recently patented by H. W. Robinson [1913] for the treatment of the tar or pitch by formaldehyde whereby the auxetics are inactivated, and an important test on a practical scale is now being made at the briquette works at Cardiff to see if this inactivation of auxetics prevents the disease occurring among the workmen.

The experiments described above, however, have suggested a simpler method of inactivation. It was noticed that aqueous extracts of tar rapidly lost their auxetic power if left exposed to the air for any considerable time. This was especially noticeable if the extracts had been made alkaline and hence the free bases unless dried or kept in an evacuated desiccator rapidly become inactive. The above facts suggested that the loss of activity was due simply to the oxidation of the auxetics and hence experiments were at once commenced with a view to rendering the tar innocuous by means of oxidation methods.

The following experiments illustrate the method finally adopted. 100 g. of tar were heated in a flask in an oil bath to 160° and a blast of air blown through. The tar was then extracted as before with acid, and the extract after neutralisation tested for auxetic action and found to be inactive in every case. A similar experiment was carried out with ozonised air and again the extract of the residue was inactive. The oxidation was quicker in this case than in the experiment where ordinary air was used. The auxetics present can therefore readily be rendered harmless by oxidation according to the method described and it is hoped shortly to try the process on a large scale. The experiments just described refer only to the auxetics, the inactivation of the kinetics being at present under investigation.

SUMMARY.

1. Methods are discussed for the separation of basic constituents of gas-works coal-tar which excite cell division.
2. The bases capable of exerting this action are found to occur in the anthracene fraction of the tar.
3. Two of these bases have been isolated as picrates but have not as yet been satisfactorily identified.
4. A method for the inactivation of these bases in tar is described.

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LXI. THE HYDROLYSIS OF GLYCOGEN BY DIASTATIC ENZYMES. II. THE INFLUENCE OF SALTS ON THE RATE OF HYDROLYSIS. (Preliminary Communication.)

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(Received Nov. 17th, 1913.)

In a recent communication [Norris, 1912] it was pointed out that samples of glycogen prepared from different animals were hydrolysed at different rates by pancreatic amylase. In view, however, of the fact that these preparations contained varying amounts of salts it seemed desirable to examine how far this might influence the rate of hydrolysis, and a quantitative study has therefore been made of the action of neutral salts on diastatic action. The results quickly showed that the experiments referred to were in no way invalidated by the varying salt content, the latter in every case being sufficient to produce the maximum degree of hydrolysis and not great enough to cause any inhibition.

The experiments have however been continued in the hope of finding some explanation of the manner in which salts exert their influence.

It has been known for some time that a dialysed amylase solution when added to a starch solution free from salts produces but little hydrolysis, and that on adding certain salts in small quantities the activity of the enzyme is restored. In spite of a considerable amount of work on this point, the explanation of this fact is not by any means clear. It is agreed that the most active salts are those of the halogen acids. With regard to sulphates, however, very divergent results have been obtained. Cole [1906, 1], for example, states that sulphates accelerate the action while Grützner [1902] maintains that magnesium and sodium sulphate are "specific poisons" for the ferment. In this connection however it must be pointed out that in one at least of Cole's experiments the addition of sulphate produced no acceleration although the concentration of the salt was similar to that used in previous experiments in which an acceleration had been obtained. These and other divergencies

may perhaps be partly explained by one of the following reasons. Firstly, the source and method of preparation of the enzyme has differed with nearly every worker. Hence while some investigators have employed solutions containing only traces of proteins, in other cases these have been present in considerable quantity. Again, in the few cases where the action has been followed quantitatively, the diastatic activity has been estimated either by Roberts' achromic point method [Roberts, 1891] or by the method of Wohlgemuth [1908, 1], and it has been shown by Evans [1912, 1] that neither of these is satisfactory. Finally, in some cases, the enzyme and starch solution employed have been by no means free from salts, the controls all showing a marked degree of hydrolysis, that is to say the observed effect was really due to a mixture of salts and not alone to the particular salt under investigation.

In the following experiments glycogen has been employed instead of starch, while the enzyme has consisted of an extract of pigs' pancreas.

Preparation of glycogen. This was obtained from dogs' liver by Pflüger's method. The crude glycogen was purified by repeated precipitation of its solution by alcohol and was finally dialysed for a week, the last three days' dialysis being against running distilled water.

Preparation of enzyme. This consisted of a Buchner extract of pigs' pancreas which was dialysed for three days, in the course of which a certain amount of protein usually separated out. The dialysed extract was then filtered till perfectly clear and diluted from ten to twenty times with distilled water.

Experimental methods. The following may be described as typical of the method employed.

A 1 per cent. or 2 per cent. solution of glycogen was as a rule used and to this was added a suitable concentration of the salt under investigation. The mixture was then brought to a temperature of 37° in a thermostat and the enzyme added. After 15 minutes and 30 minutes hydrolysis, 20 cc. of the mixture were removed and the sugar immediately estimated by Bertrand's method. The sugar solution was added directly to the alkaline copper mixture and hence the hydrolytic action stopped instantaneously. The strength of enzyme used was adjusted so that the readings taken fell on the linear portion of the hydrolysis curve. [See Evans, 1912, 2 and Norris, 1912.] The salts employed were nearly all Kahlbaum's "for analysis with certificate of guarantee."

Effect of dialysis on glycogen hydrolysis.

As in the case of starch the result of dialysis of both enzyme and glycogen resulted in almost complete inactivation. Similarly the hydrolytic power was again restored by the addition of certain salts.

Influence of salts.

Under this heading neutral salts only are considered. The rate of hydrolysis is of course greatly influenced by any change in the reaction of the medium, but this point has been dealt with in a previous communication [Norris, 1912].

Hydrogen ion determinations made on glycogen solutions containing varying concentrations of sodium chloride showed that the reaction of the medium was not altered by the presence of this salt in the concentrations employed, hence the accelerating effect of sodium chloride is not due to this cause.

Sodium chloride.

A series of mixtures was made up each containing 1 per cent. glycogen and a concentration of sodium chloride ranging from zero to 0.003 N. These were in turn incubated with 1 cc. of a dilute enzyme preparation and hydrolysis allowed to proceed for 15 minutes. The sugar in 20 cc. of each solution was then estimated.

The results are shown in Fig. 1 where the abscissae represent the cc. of 0.1 N NaCl in 100 cc. of the mixture and the ordinates the cc. of KMnO_4 used in the sugar estimation.

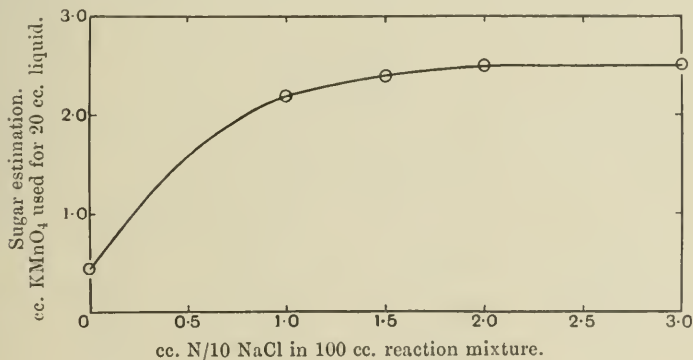


Fig. 1.

The results show that there is a rapid increase in the hydrolysis with increasing NaCl content until the latter reaches a concentration of about 0.002 N (0.012 per cent.). This value is in close agreement with Cole's results with starch and ptyalin.

On further addition of salt there is at first no change in rate, but with high concentration a slight retardation may be produced.

An investigation was next undertaken to decide whether the value of this optimum concentration (0.002 N) would be changed by alteration in the concentration of either glycogen or enzyme. On this point results have been somewhat contradictory but it seems probable that the glycogen concentration has but little influence on the amount of salt required to produce the maximum rate of hydrolysis. The optimum salt content for 2 per cent. glycogen has usually been about 0.002 N, that is to say the same as for 1 per cent. glycogen.

On increasing the amount of enzyme, however, a higher concentration of NaCl is usually required. This is in agreement with the results of Cole [1906, 1] and Starkenstein [1910]. The latter, working with starch, states that the amount of NaCl necessary to give the maximum rate of hydrolysis varies *directly* with the concentration of enzyme and has even based on this a method for the estimation of diastase in animal organs [Starkenstein, 1912]. The results of my experiments do not point to such a simple relationship, though as already stated more NaCl is usually required with increased concentration of enzyme. It must be remembered that it is usually in the enzyme that impurities such as proteins etc. will be found and the latter may begin to play an important part when the amount of enzyme present is not small.

The following table shows the results of one experiment in which the concentration of enzyme was twice that usually employed.

TABLE I.

Determination of NaCl optimum with 1 per cent. glycogen and high enzyme concentration.

Experiment	Concentration of NaCl	Percentage hydrolysis	
		15 mins.	30 mins.
A	0	5.5	9.20
B	0.002 N	15.1	26.5
C	0.004 N	16.5	28.0
D	0.006 N	18.3	29.8
E	0.01 N	18.3	29.5

In this case therefore by doubling the concentration of enzyme the optimum salt concentration was raised from 0.002 N to 0.006 N, that is to say three times.

In other experiments however very much lower results were obtained and there seems to be some unknown factor concerned. The point is still under investigation.

Comparison of sodium chloride with other salts.

Chlorides of different metals.

Table II shows the accelerating effect of the chlorides of sodium, potassium, calcium, barium and magnesium.

These were added in insufficient quantity to produce the optimum rate of hydrolysis, so that any variation in their accelerating power could be detected.

TABLE II.

*Accelerating power of various chlorides. 1 per cent. glycogen.
Concentration of salt = 0.0005 N.*

Salt		Percentage hydrolysis	
		15 mins.	30 mins.
	NaCl	10.83	18.76
	KCl	10.61	18.8
	CaCl ₂	10.70	—
	BaCl ₂	10.61	17.6
	MgCl ₂	10.61	18.52
Control	0	0.68	1.14

All the above salts are therefore of equal accelerating power, that is to say the cation exerts practically no influence on the reaction. This is in agreement with the results of Starkenstein [1910]. Cole [1906, 1] considered that the anion accelerated while the cation depressed the action. If the latter were correct, however, one would expect the chlorides containing a divalent cation to be less active than sodium or potassium chloride and as shown this is not the case. The view that the anion is the more important factor is further strengthened by a comparison of the accelerating power of chlorides, bromides and iodides. The results given in Table III confirm those of previous investigators, namely, that the acceleration decreases in the order given, the drop from bromide to iodide being much greater than that from chloride to bromide.

TABLE III.

Comparison of KCl, KBr and KI. (Concentration of salt = 0.0005 N.)

Salt	Percentage hydrolysis	
	15 mins.	30 mins.
KCl	10.61	18.80
KBr	8.31	15.60
KI	2.5	3.70
Control	0.68	1.40

Influence of sulphates.

The influence of three sulphates has been investigated but it has been found that they have no accelerating power at all; these results are therefore in agreement with those of Wohlgemuth [1908, 2] but in opposition to those of Cole [1906, 1]. The latter however found that sulphates were much less effective than the halogen compounds. These results are of some interest, as one would expect if the anion were the factor concerned, that the divalent anion would be extremely potent. The situation is complicated, however, as pointed out by Cole, by the fact that sodium sulphate in moderate dilutions chiefly dissociates into Na^+ and NaSO_4^- in which case the anion is monovalent.

On the other hand it has not been found that sulphates have any depressing action [cf. Grützner, 1902], nor do they hinder the acceleration produced by NaCl etc.

TABLE IV.

Influence of sulphates. 1 per cent. glycogen.

		Concentration of salt	Percentage hydrolysis	
			15 mins.	30 mins.
1.	0	Control	1.60	5.04
	Na_2SO_4	0.002 N	1.65	5.0
	Na_2SO_4	0.01 N	1.55	5.0
	MgSO_4	0.001 N	1.60	—
2.	MgSO_4	Each		
	+	0.001 N	9.10	16.04
	NaCl			
	NaCl	0.001 N	9.20	16.10

For the sake of comparison the results obtained with different salts have been collected in Table V.

TABLE V.

Comparison of various salts. 1 per cent. glycogen containing 0.0005 N salt.

Salt	Percentage hydrolysis		Remarks
	15 mins.	30 mins.	
0 (Control)	0.68	1.14	
NaCl	10.83	18.76	Halogen salts. Both ions monovalent.
KCl	10.61	18.8	
KBr	8.31	15.6	
KI	2.5	3.7	
CaCl ₂	10.70	—	Halogen salts with divalent kation.
BaCl ₂	10.61	17.6	
MgCl ₂	10.61	18.5	
KNO ₃	2.75	5.08	Both ions monovalent.
La(NO ₃) ₃	2.75	4.94	Trivalent kation.
Na ₂ SO ₄	0.55	1.0	Divalent anion.
K ₂ SO ₄	0.64	1.20	" "
MgSO ₄	0.70	—	Both ions divalent.

It will be seen from the results tabulated above that the only salts of those tried which have a powerful accelerating action are those of the halogen acids, although nitrates have a small influence. It is also clear that the anion is much more concerned in the reaction than is the kation. Further it is probable that the action of the salts is chiefly confined to the enzyme, for it has been shown by Cole [1906, 2] that in the action of invertase, where the substrate is not a colloidal solution, salts have again a powerful influence. In this case, however, the action is reversed, that is to say, the hydrolysis is retarded by chlorides.

If the function of the anion be simply to alter the charge on the enzyme, it is difficult to understand why sulphates have no accelerating effect, for sulphates discharge a ferric hydroxide solution much more readily than chlorides. The fact that the number of salts producing an acceleration is so restricted, however, points to some other explanation.

In the meantime it seems desirable to examine separately the effect of salts on the glycogen and enzyme respectively from the point of view of adsorption and charge, and experiments on these lines are in progress but are not as yet sufficiently advanced to furnish an explanation. The action of a further series of salts is also being investigated.

SUMMARY.

(1) A dialysed glycogenase (pancreatic) solution has practically no hydrolysing action when added to a dialysed glycogen solution.

(2) The activity of the enzyme is restored by the addition of small quantities of certain salts.

(3) The most powerful of these are the salts of the halogen acids, the activity diminishing in the order chlorides, bromides, iodides; nitrates have also a slight accelerating action.

(4) Sulphates do not restore the activity of a dialysed enzyme solution, neither are they inhibitors [cf. Grützner, 1902].

(5) The concentration of salt required to produce a maximum degree of hydrolysis rises with increasing enzyme concentration but appears to be independent of the glycogen concentration within the limits tried.

(6) The anion is probably the part of the salt concerned in the acceleration, the nature of the kation (valency) having no influence.

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LVII. ON THE BEHAVIOUR OF AMYLASE IN THE PRESENCE OF A SPECIFIC PRECIPITATE.

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(Received Nov. 10th, 1913.)

The complement-binding phenomenon which takes place in the presence of antigen and antibody has become of great practical importance in the recognition of disease. The difficulties of the test are great, owing to the fact that five different, chemically impure, and little known substances must be employed: antigen, antiserum, complement, amboceptor, and red corpuscles. The last three components are very troublesome to obtain, and it is not surprising that many efforts should be made to minimise this trouble, and to lessen the difficulties which are necessarily dependent on such a complicated process. The most attractive simplification would be one in which all these three components of the haemolytic system, viz. complement, amboceptor, and red corpuscles, are entirely done away with. This would be possible if some substance could be found which would become fixed, like complement, by the antigen-antibody mixture, but whose absence could be tested for more easily.

Like complement, animal ferments seem to be very rapidly absorbed by coagulated colloids and even by organic precipitates of different kinds. The ferment shows some selective action in this tendency. Pepsin absorbed into egg-white [Danwe, 1905, p. 426] or pepsin, ptyalin, etc. into collodion [Porter, 1910, p. 382] can be recovered in some degree by a solution of the particular substance which they digest. As ferments can only be recognised through their effects, this possibility of partial recovery presents the chief objection to the use of ferments as substitutes for complement.

Most ferments are powerfully influenced by the presence of serum. Proteolytic ferments are inhibited by serum, probably because their attraction to the serum proteins, even in a dissolved condition, is already so great. On the other hand other ferments, such as starch-splitting ferments and lipases, are markedly accelerated by the presence of serum.

Hailer [1908, p. 280] first made the attempt of absorbing a ferment by means of a specific precipitate. He chose rennet, with sheep's serum as antigen, and the serum of a rabbit immunised against sheep's protein as antibody. This choice was perhaps unfortunate, as not only rennet but also antirennet have been described in serum [Fuld and Spiro, 1900, p. 141]. Also, as I have pointed out, rennet suffers very little from dilution, when the dilution has been freshly made. Rennet has indeed acted perfectly in my hands [1911, p. 394] at a dilution of 1/340,000. It can therefore be almost entirely absorbed without any evidence of the fact. On this account I thought it advisable to attempt the absorption with another ferment. Pepsin and trypsin are inactivated by serum; I therefore chose amylase. Szumowski [1898, p. 162] has proved that amylase is absorbed by fibrin. It is much less easily absorbed by serum proteins in their natural state, as serum is itself amylolytic. The advantages of the ferment were therefore as follows:

(1) It is present in serum, so that, were the absorption successful, no further addition of ferment to the antiserum would be required.

(2) It is apparently not absorbed by serum proteins in their normal state, i.e. before the union of antigen and antibody.

(3) It loses rapidly on dilution, so that even a small loss could be measured.

METHOD.

Ferment, guinea-pig-serum, saliva, and taka-diasatase.

Antibody, serum of rabbits immunised against egg-white or against horse-serum.

Antigen, egg-white or horse-serum.

The ferment, in as small bulk as possible, i.e. 0.05 cc. saliva, or 0.15 cc. diluted taka-diasatase, was added to 0.15 cc. immune or normal serum, and 0.15 cc. of the various dilutions of egg-white or horse-serum, the mixture being left at room temperature overnight. Next morning 3 cc. of a 1% soluble starch solution were added, and the tubes were placed at 37° for 5 to 20 minutes, or in the case of guinea-pig-serum for a half to one and a half hours. The mixtures were then tested for sugar by means of Fehling's solution, and for dextrans by means of iodine. On account of the protein present, use was made of the dialysed iron method, which had the double advantage of removing both protein and ferment. When the mixtures were taken from the incubator, they were made up to a bulk of 5 or 6 cc. with physiological saline solution, and 0.5 cc. saturated salt solution, and 0.5 cc.

B.P. Liquor Ferri Dialysatus were added to each and the resulting precipitate immediately filtered off. A measured amount of the perfectly clear and inactive filtrate was then tested quantitatively for sugar. The colour given by the iodine test in the filtrate was clear and permanent.

The difference between the effect of immune and normal serum on amylase in the presence of antigen was very slight, but on account of the accuracy of the method and the number of times the experiment has been repeated, it may be taken as the expression of a genuine though very partial absorption.

Absorption experiment.

A. Immune serum. Rabbit immunised against egg-white.

(To each tube were added 0.05 cc. saliva, 0.15 cc. anti-egg-serum, 0.15 cc. diluted egg-white, method as above.)

Dilution of egg-white	Time at 37° with starch	Cc. of Fehling sol.	Iodine solution 0.5 cc.
1/10	10 minutes	0.2	Dark mauve.
1/50	"	0.225	"
1/100	"	0.225	"
1/250	"	0.25	"
1/500	"	0.25	Light mauve.
NaCl	"	0.3	Pale pink.
1/10	20 minutes	0.25	Dark mauve.
1/50	"	0.25	Medium mauve.
1/100	"	0.25	" "
1/250	"	0.275	Medium light mauve.
1/500	"	0.3	Light mauve.
NaCl	"	0.35	Very pale.

B. Normal rabbit-serum.

(To each tube were added 0.05 cc. saliva, 0.15 cc. normal serum, 0.15 cc. diluted egg-white, method as above.)

1/10 to 1/500	10 minutes	0.3	All pale.
NaCl	"	0.3	Pale.
1/10 to 1/500	20 minutes	0.375	"
NaCl	"	0.375	"

When serum is used as antigen a curious phenomenon may be noticed. In this case all three ingredients, antigen, antibody, and saliva, contain amylase from three separate species, and this gives rise to an acceleration which is decidedly beyond the sum of the three separate activities. Although the antiserum was heated at 56° to reduce its amylolytic power, it was still able to exert an accelerating influence. In the following experiment, where the antigen is horse-serum, acceleration is to be seen and must be allowed for. In spite of it, absorption can be observed if the "immune" and "normal" columns are compared.

A. *Rabbit-serum*, immune to horse-serum.

(To each tube were added 0.05 cc. saliva, 0.15 cc. anti-horse-serum, 0.15 cc. diluted horse-serum, method as above.)

Dilution of horse-serum	Time at 37° with starch	Ccs of Fehling sol.	Colouration with iodine solution
1/10	10 minutes	0.5	Red mauve.
1/50	"	0.45	"
1/100	"	0.4	"
1/250	"	0.4	Same, but deeper.
1/500	"	0.35	Mauve.
NaCl	"	0.35	"
1/10	20 minutes	0.5	Pale pink.
1/50	"	0.5	"
1/100	"	0.45	"
1/250	"	0.4	Mauve.
1/500	"	0.35	"
NaCl	"	0.35	"

B. *Normal rabbit-serum*.

(To each tube were added 0.05 cc. saliva, 0.15 cc. normal rabbit-serum, 0.15 cc. diluted horse-serum, method as above.)

1/10	10 minutes	0.55	Colourless.
1/50	"	0.55	"
1/100	"	0.5	"
1/250	"	0.4	Pale.
1/500	"	0.35	Mauve.
NaCl	"	0.35	"
1/10	20 minutes	0.6	Colourless.
1/50	"	0.6	"
1/100	"	0.55	"
1/250	"	0.45	"
1/500	"	0.4	Pale.
NaCl	"	0.35	Mauve.

Taka-diastase displayed no tendency whatsoever to become absorbed by a specific precipitate. This is interesting, as I have noticed before [1910, p. 386] that while ptyalin was inactivated by collodion membranes in a day, taka-diastase was hardly affected in a month's time.

BEHAVIOUR OF SERUM AMYLASE ON PRECIPITATION WITH CARBON DIOXIDE.

Fresh guinea-pig-serum, diluted to 1/10 in water, was saturated with carbon dioxide, and the globulins separated and redissolved in saline solution, after centrifuging. The upper fluid taken from the centrifuge had its isotonicity restored by sodium chloride, and both portions were tested for amylase. The ferment was found to be almost unaffected by the precipitation, practically the whole ferment remaining free in the upper fluid.

Time 1½ hours	Cc. of Fehling sol.	Colouration with iodine
1 cc. serum 1/10 + 1 cc. NaCl 0·85 %	0·5	Brown.
1 cc. precipitate (1/10) + 1 cc. NaCl 0·85 %	0·05	Dull, dark.
1 cc. upper fluid + 1 cc. NaCl 0·85 %	0·4	Brown.
1 cc. serum 1/10 + 1 cc. precipitate	0·475	„
1 cc. serum 1/10 + 1 cc. upper fluid	0·65	Colourless.
1 cc. precipitate + 1 cc. upper fluid	0·425	Brown.

Although these experiments have been attended with little success, I am venturing to record them as of some small theoretic interest, and partly to recommend a good method for testing amylolytic action in the presence of serum.

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XXIV. QUANTITATIVE RELATIONS IN
CAPILLARY ANALYSIS.

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When a dispersed system is allowed to fall on filter- or blotting-paper or to rise up into a strip of such a paper, one observes that the dispersed phase remains behind the medium of dispersion, which is in most cases water. This phenomenon, which involves an increase in the concentration of the dispersed phase in the paper, can be used for the qualitative testing of the nature of the substance as has been shown by the capillary analytical experiments of Goppelsroeder [1909].

Holmgren [1908] tried to establish a formula, which gives a quantitative relation between the extents of the dispersion medium and the dispersed phase. He experimented with diluted acids, especially with hydrochloric acid, by allowing the latter to drop on blotting-paper. Using Congo-red as indicator, he was able to show how far the acid was spread over the paper compared with the water. If a good and homogeneous blotting-paper is used, the figure caused by the diffusion of the drop approaches a circle.

Holmgren supposed that the relation between the water-zone, the largest radius of which may be R , and the inner circle of radius r caused by the acid, is constant for each concentration, so that the quotient of two different concentrations is equal to the quotient of these relations. Therefore, as $[\pi R^2 - \pi r^2]$ is the surface of the water-ring, the following relation exists according to Holmgren :

$$\frac{C}{C_1} = \frac{\frac{r^2}{R^2 - r^2}}{\frac{r_1^2}{R_1^2 - r_1^2}} \dots\dots\dots (1).$$

If we signify by P that concentration for which

$$\frac{r_1^2}{R_1^2 - r_1^2} = 1,$$

the following equation results:

$$C = P \cdot \frac{r^2}{R^2 - r^2} \dots\dots\dots(2),$$

or

$$P = C \cdot \frac{R^2 - r^2}{r^2} \dots\dots\dots(3).$$

If, therefore, the concentration C and the radii of the circles produced on blotting-paper by any drop of the acid are known, P is determined from equation (3). Holmgren supposed P to be constant for the same paper and independent of the concentration. He called P the paper constant and was then able to determine any other concentration by the equation (2), but with the limitation that this method was not to be used except for very dilute solutions between 2.0 and 0.01 %. The results of Goppel-roeder's experiments, which give only the measurements of capillary height in strips of filter-paper, can also be treated by this formula of Holmgren, if instead of the height-measurements their squares are taken.

Skraup [1909, 1 and 2] and his co-workers were able to confirm the applicability of this formula for capillary-analysis. On examining the amount of free hydrochloric acid in the gastric juice by this method, I found [Schmidt, 1913] that the results obtained agreed very closely with those obtained by titration. Later some doubts arose in my mind as to the correctness of Holmgren's formula and occasioned me to undertake the following investigation.

TECHNIQUE OF EXPERIMENTS.

I experimented exclusively with hydrochloric acid, an exactly adjusted normal solution of which was taken as the basis for the following dilutions between 2.0 and 0.05 %. One drop of these solutions was allowed to fall on blotting-paper from a short distance. Much attention was paid to insure that the drop had always the same volume. With an ordinary pipette it was not possible to get the volumes of the drops as exact as was desirable. I therefore used the dropping surface of Traube's stalagmometer, which gives exactly equal drops, if the temperature be kept constant and the instrument be not allowed to deviate from the vertical position. Blotting-papers are much better fitted for capillary analysis than filter-paper, but they must not be too thick and must have a smooth surface and a most homogeneous structure. I obtained a series of different blotting-papers from the firm of C. Schleicher und Schüll, Düren i. Rheinland. The papers marked Nos. 123

and 117 proved to be specially suitable for any purpose. The papers were stained by floating them rapidly through an alcoholic solution of Congo-red (0.04%) or methyl-orange (0.1%). In order to prevent the water from evaporating or at least to reduce the evaporation to a minimum, as soon as the drop was absorbed the paper was placed between two glass plates and a weight laid on the plate to avoid any wrinkling. The plates were then put on a small frame under a glass globe, the interior of which was kept saturated with moisture by means of wetted filter-paper below. To neglect the evaporation leads to false conclusions. The radii were measured in millimetres by the use of transmitted or reflected light, and since the figures of the larger spots are mostly elliptical owing to the direction of the fibres in the structure of the paper, the main axes as well as two oblique axes were measured, so that the given result is the average of the measurement of 4 diameters. The number of figures examined to determine any single point is in most cases at least 4. The measurement took place after exactly one hour, the process having then practically reached a standstill. In reality one may sometimes observe an increase of the radii even after 24 hours, but the amount of it is too insignificant to be noted, especially if only one drop is taken.

Referring to dilutions of acid under 0.1% the measurement must take place earlier than one hour especially when Congo-red is used, because the blue colour disappears after a certain time, owing to chemical action.

EXPERIMENTS.

The mass of one drop given by a stalagmometer at the temperature of 18–20° is indicated by a . If the drop consisted of distilled water, its weight was found to be 0.1160 gr., and if it consisted of HCl of a concentration between 2.0 and 0.05 %, I found the average weight of a drop 0.116 gr., the differences of the specific gravity in so highly diluted acids being so small that they can be neglected without introducing an error which exceeds the error of observation.

Therefore, putting the volume equal to the weight (in case of dilute HCl) I give a the value of 0.116 gr. Supposing m parts of hydrochloric acid to be present in the drop a , the concentration is given by $C = \frac{m}{a}$ by means of which m is easily calculated.

Under the above described conditions, a series of observations was made, of which the following may be reproduced:

TABLE I.

Paper No. 123. $a=0.116$ gr.

Indicator:		Congo-red						Methyl-orange			
Number of drops:		1 a		2 a		3 a		1 a		2 a	
HCl %	m	R	r	R	r	R	r	R	r	R	r
2.0	2.32	23.12	21.87	32	29.37	37.87	34.25	23.12	21.62	29.81	26.43
1.0	1.16	23.5	19.62	32.37	26.5	38.5	31.25	23.37	19.56	31.75	26
0.75	0.87	24	18.37	32.37	24.5	38.7	29.5	23.37	18	32	24.25
0.5	0.58	23.62	16.56	31.87	22.18	37.8	26.5	24	16.87	32.25	22.43
0.4	0.464	23.87	15.75	32.75	21.5	38.2	22.12 (?)	23.62	15.56	32.37	20.75
0.3	0.348	23.62	14.63	32.25	19.68	37.0	23.5	23.62	14.75	31.62	19.56
0.2	0.232	23.37	12.37	31.5	17.12	37.3	20.62	23.62	12.25	32.12	17.12
0.1	0.116	23.5	10	31.62	13.5	37.8	16.62	23.62	10.75	32	13.75
0.05	0.058	23.7	8.62	—	—	—	—	23.43	—	—	—
Means of R		23.48		32.09				23.53		31.74	

TABLE II.

Paper No. 117. $a=0.116$ gr. Indicator: Congo-red.

Number of drops:		1 a		2 a		3 a	
HCl %	m	R	r	R	r	R	r
1.0	1.16	26.5	24.25	36.0	32.41	42.25	37.5
0.75	0.87	26.85	22.40	35.75	30.1	42.5	35.5
0.5	0.58	26.68	20.62	35.43	27.75	41.62	33.18
0.4	0.464	26.87	20.5	35.43	27.5	43	33.0
0.3	0.348	25.87	18.85	35.87	25.2	42.37	30.3
0.2	0.232	26.25	16.37	36.0	22.81	42.0	26.75
0.1	0.116	26.25	13.63	35.75	18.5	42.25	22.75
0.05	0.058	26.75	—	—	—	—	—
Means of R		26.50		35.75		42.28	

TABLE III.

Paper No. 123. $a=0.0311$ gr.

Indicator:		Congo-red		Methyl-orange	
HCl %	m	R	r	R	r
1.0	0.311	14.75	13.29	—	—
0.5	0.155	15.1	12.5	12.5	9.3
0.4	0.124	14.65	9.4	12.8	8.9
0.3	0.0933	14.84	8.85	12.7	8.25
0.2	0.0622	15.15	7.95	12.8	7
0.182	0.0566	13.3	6.4	12.9	6.36
0.1	0.0311	14.26	6.0	13.1	6
Means of R		14.6		12.8	

On the assumption that the aggregation of acid in the inner circle is of the nature of an adsorption process, the relationship between the two variables m and r must be expressible by the exponential equation

$$r = \beta m^n.$$

It is highly probable also that the concentration of the acid or that of the hydrogen ions is continuously decreasing from the centre to the periphery of the spot-system in the case of any drop of a certain concentration falling on the paper. I suppose then, that r indicates that point of hydrogen ion concentration which corresponds with the sensibility of the indicator. But owing to the impossibility of proving experimentally the gradual decrease of the hydrogen ion concentration in the paper between the centre and r , my supposition remains an hypothesis. To show how in different concentrations the relation between m and r is expressible by the equation $r = \beta m^n$, we use the graphic method in constructing the m - r -curve Fig. 1.

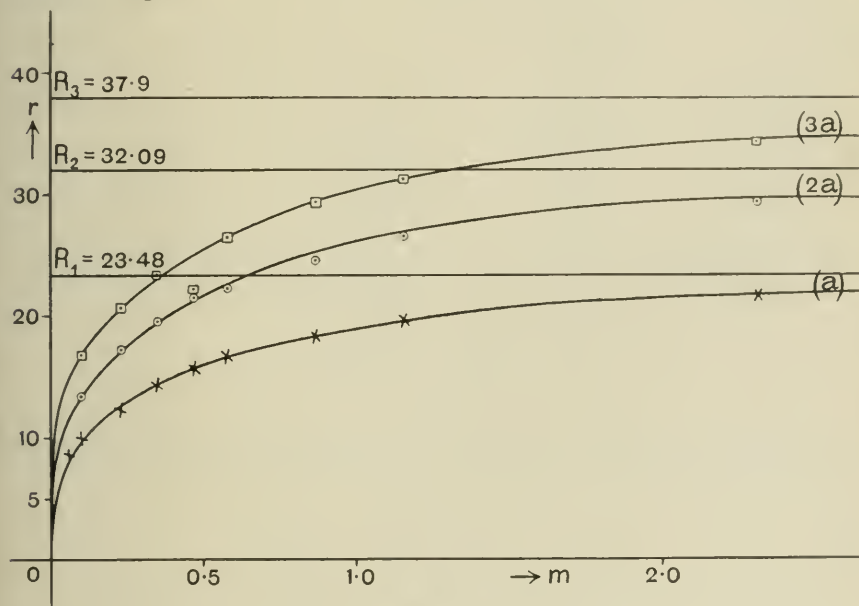


Fig. 1.

The m - r -curve rises very steeply in the beginning and then runs nearly parallel to the m -axis, asymptotically approaching a straight line, of which the equation is $r = R$. The difference $(R - r)$ is identical with the breadth of the water-zone and the latter is of course equal to R in case of distilled water, when m or $C = 0$. $(R - r)$ decreases with increase in the amount of m . Theoretically, the decrease of $(R - r)$ is infinite but in reality m reaches very soon such an amount, that the water-zone is no longer visible. When the latter effect occurs with one drop, the phenomenon can still be obtained by increasing the volume of the drop or augmenting the number of drops, but the errors of observation are also increasing and in such a

degree that for concentrations higher than 2.0% the method of Goppelsroeder is preferable, which, as already mentioned, involves the measurement of the capillary height in strips of filter-paper.

That R is in reality the constant, which it seems to be in the experimental data, and that R depends—in the same paper—only upon the volume of the drop and not upon the concentration, will be shown later.

By taking the logs. of the equation $r = \beta \cdot m^p$, we get

$$\log r = p \cdot \log m + \log \beta.$$

This is the equation of a straight line, which is very nearly approached by plotting the values of $\log r$ and $\log m$, as is shown in Fig. 2.

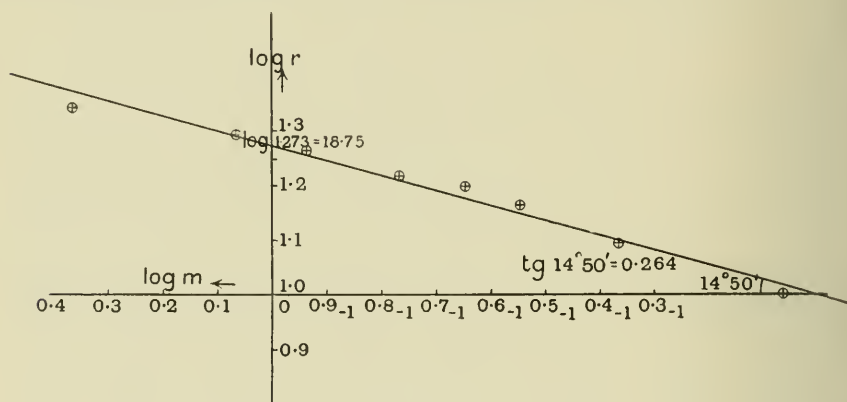


Fig. 2.

By graphic interpolation according to Freundlich's [1907] procedure the values of β and p are found to be

$$p = 0.264$$

and

$$\beta = 18.75.$$

In the following table the values are calculated by the equation

$$r = 18.75m^{0.264} \dots\dots\dots(4).$$

TABLE IV.

Paper 123. Congo-red. 1 drop ($a = 0.116$ gr.). $r = 18.75m^{0.264}$.

$C = \% \text{ HCl}$	m	r calculated	r observed	Δ calc. - obs.
2.0	2.32	23.41	21.87	-1.54
1.0	1.16	19.49	19.62	+0.13
0.75	0.87	18.08	18.37	+0.29
0.5	0.58	16.31	16.56	+0.15
0.4	0.464	15.12	15.75	+0.63
0.3	0.348	14.19	14.63	+0.44
0.2	0.232	12.74	12.37	-0.37
0.1	0.116	10.61	10	-0.61
0.05	0.058	8.84	8.62	-0.22

Although the agreement is fairly good, a further calculation of β and p was made by the following procedure in order to diminish the differences.

HCl %	$100 \times m$	$100 \times r$	$\log m$	$\log r$
2.0	232	2187	2.36549	3.33985
1.0	116	1962	2.06446	3.29248
0.75	87	1837	1.93952	3.26411
0.5	58	1656	1.77085	3.21906
0.4	46.4	1575	1.64652	3.19728
0.3	34.8	1463	1.54158	3.16524
0.2	23.2	1237	1.36549	3.09237
0.1	11.6	1000	1.06446	3
0.05	5.8	862	0.76343	2.93551
Mean			1.613533	3.167322

Deviations from means :

m_1	r_1	$m_1 \cdot r_1$	$(m_1)^2$
+ 0.75195	+ 0.172528	0.12957	0.56543
+ 0.45092	+ 0.125158	0.056432	0.20333
+ 0.32598	+ 0.096788	0.031551	0.051067
+ 0.15731	+ 0.051738	0.0081424	0.024746
+ 0.032987	+ 0.029958	0.00098825	0.0010881
- 0.071953	- 0.002082	0.000149806	0.0051772
- 0.248043	- 0.054952	0.013630	0.061523
- 0.549073	- 0.167322	0.091871	0.30147
- 0.850103	- 0.231812	0.19705	0.72267

$$p = \frac{\sum (m_1 \cdot r_1)}{\sum (m_1)^2} = \frac{0.529384}{1.9365013} = 0.2733.$$

The value 0.273 for the exponent p gives the following values for

$$\log \beta_1 = \log r - 0.273 \log m.$$

$\log \beta_1$	and for β_1
2.694072	494.4
2.728883	535.6
2.73462	542.7
2.73561	544.0
2.74778	559.4
2.74438	555.1
2.711959	524.3
2.70940	512.1
2.72709	533.4

As the figures for r and m have been multiplied by 100, the values of $\log \beta$ in the equation

$$r = \beta \cdot m^p$$

are calculated from the following equation :

$$\log \beta = \log \beta_1 + 2 \cdot (0.273) - 2.$$

These values of $\log \beta$ are therefore the following :

$\log \beta$	β
1.25007	17.78
1.27488	18.83
1.28062	19.08
1.28161	19.12
1.29378	19.66
1.29038	19.51
1.26559	18.43
1.25540	18.00
1.27309	18.75

Mean of β 18.88

It results therefore for the relation between r and m the equation

$$r = 18.88 \cdot m^{0.273} \dots\dots\dots(5).$$

Thus substituting the calculated values for β and p the following equation was obtained:

$$r = 18.88 \cdot m^{0.273},$$

which gives a far better agreement between calculation and observation, as is shown in the following table:

TABLE V.

Paper 123. Indicator: Congo-red. One drop. $a=0.116$ gr. $r=18.88 \cdot m^{0.273}$.				
$C=\%$ HCl	m	r calculated	r observed	Δ
2.0	2.32	23.75	21.87	-1.88
1.0	1.16	19.66	19.62	-0.04
0.75	0.87	18.17	18.37	+0.2
0.5	0.58	16.34	16.56	+0.22
0.4	0.464	15.11	15.75	+0.64
0.3	0.348	14.15	14.63	+0.48
0.2	0.232	12.67	12.37	-0.3
0.1	0.116	10.48	10.0	-0.48
0.05	0.058	8.677	8.62	-0.05
Idem, but $a=0.0311$ gr.				
1.0	0.311	13.72	13.29	-0.43
0.5	0.155	11.09	12.5	+1.41
0.4	0.124	10.67	9.4	-1.27
0.3	0.0933	9.880	8.85	-1.03
0.2	0.0622	8.845	7.95	-0.89
0.182	0.0566	8.620	6.4	-2.42
0.1	0.0311	7.320	6	-1.32

The reason why the second part of the Table V does not show so good an agreement is the inexact measurement of drops by using an ordinary pipette instead of a stalagmometer. By increasing the number of drops, so that instead of a , $2a$ or $3a$ were taken of the same concentration $C=\frac{m}{a}$, the radii of the corresponding spots must be

$$r\sqrt{2}, \quad r\sqrt{3},$$

or, generally speaking, if $[na]$ is taken as the dropping mass, the corresponding

radius is r/n , r being the radius for a . Therefore, if the radii found for a are supposed to be fixed, the radii for $2a$ or $3a$ can be calculated. The following table gives the results:

TABLE VI.

Paper 123. Indicator: Congo-red. $a=0.116$ gr.

$C=\%$ HCl	a	$2a$		$3a$	
		r_2 calculated	r_2 observed	r_3 calculated	r_3 observed
2.0	21.87	30.83	29.375	37.63	34.25
1.0	19.62	27.66	26.5	33.98	31.25
0.75	18.37	25.90	24.5	31.81	29.5
0.5	16.56	23.34	22.185	28.68	26.5
0.4	15.75	22.20	21.5	27.27	22.125 (?)
0.3	14.63	20.63	19.685	25.33	23.5
0.2	12.37	17.44	17.125	21.42	20.625
0.1	10.0	14.14	13.5	17.32	16.625
0.05	8.62	—	—	—	—

The agreement between the calculated and observed values for r is still fairly good for $2a$. But by increasing the number of drops beyond 2, the experimental error increases, because the figure produced on paper loses the resemblance to a circle, so that the average of r is determined with more arbitrariness. By using the equation (5)

$$r = 18.88 \cdot m^{0.273}$$

for cases where, generally speaking, (na) was taken, m becomes (nm) . The values of r_2 for $2m$ and of r_3 for $3m$ are shown in the following table:

TABLE VII.

Paper No. 123. Indicator: Congo-red. $a=0.116$ gr. $r\sqrt{n}=18.88 (nm)^{0.273}$.

$C=\%$ HCl	$2m$	$\log 2m$	r_2 calculated	r_2 observed	Δ observed	Δ r_2 calculated
					— calc.	— $r_2=r_1\sqrt{2}$
2.0	4.64	0.66652	28.71	29.37	+0.66	-2.12
1.0	2.32	0.36549	23.75	26.5	+3.25	-3.91
0.75	1.74	0.24055	21.96	24.5	+2.54	-3.94
0.5	1.16	0.06446	19.66	22.18	+2.52	-3.68
0.4	0.928	0.96755 - 1	18.38	21.5	+3.12	-3.82
0.3	0.696	0.84261 - 1	17.10	19.68	+2.58	-3.53
0.2	0.464	0.66652 - 1	15.31	17.12	+1.81	-2.13
0.1	0.232	0.36549 - 1	12.67	13.5	+0.83	-1.47

$C=\%$ HCl	$3m$	$\log 3m$	r_3 calculated	r_3 observed	Δ observed	Δ r_3 calculated
					— calc.	— $r_3=r_1\sqrt{3}$
2.0	6.96	0.84261	32.06	34.25	+2.09	-5.57
1.0	3.48	0.54158	26.53	31.25	+4.72	-7.45
0.75	2.61	0.41664	24.53	29.5	+4.97	-7.28
0.5	1.74	0.24055	21.96	26.5	+4.54	-6.72
0.4	1.392	0.13364	20.53	22.12 (?)	+1.59 (?)	-6.74
0.3	1.044	0.01870	19.10	23.5	+4.4	-6.23
0.2	0.696	0.84261 - 1	17.10	20.62	+3.52	-4.32
0.1	0.348	0.54158 - 1	14.15	16.62	+2.47	-3.17

The deviations of the calculated figures of r from those obtained by observation are so considerable as to make it clear that the volume used for capillary-analytic purposes must not exceed a certain quantity. But the deviation of the calculated values $r_1 \sqrt{n}$ and $18.88m^{0.273}$ must be due to the inexactness of the constant in the equation $r = \beta m^p$ and to the experimental errors for r_1 . In reality the relation

$$r_1 \sqrt{n} = \beta (n \cdot m^p) \dots\dots\dots(6)$$

must exist.

THEORETICAL PART.

On detailed consideration of the processes taking place in the paper, it becomes evident that an increase of concentration of the hydrochloric acid has taken place.

If C signify the original concentration and C_1 the resulting concentration in the coloured spot, the relation between these concentrations may be deduced in the following way. The paper is supposed to be previously stained either with 0.04% alcoholic Congo-red solution, or with 0.1% alcoholic methyl-orange solution, and the staining to be done in the same manner, the paper not being allowed to adsorb much of the dye, a process which is at room temperature only a matter of time, as has been shown by W. M. Bayliss [1906]. Observation shows that stained paper differs slightly in quality from unstained, a fact to which reference will be made later.

Let $[a]$ be the constant quantity of the drop and $[na]$ the quantity of hydrochloric acid of the concentration $C = \frac{m}{a}$, dropping on a piece of blotting-paper which is supposed to be always of the same quality. The quantity $[na]$ of the acid may spread over the paper, so that the acid reaches from the centre to the distance $r \sqrt{n}$, and the largest radius of the water-zone may be $R \sqrt{n}$, if r and R are the corresponding values for $[a]$. In case of another drop-volume, the weight being g' , the radius becomes $r \sqrt{\frac{g'}{g}}$, g being 0.116 gr.

As the acid constituent of $[na]$ is in the interstices of the paper, the volume $[na]$ must be equal to the volume of the paper, the air being included, minus the specific volume of the paper-material itself in the same space. The volume occupied by $[na]$ is $\pi n R^2$ and the specific volume of the paper-material is its weight divided by its specific gravity s . The weight of a portion of paper of square millimetre surface and thickness δ is indicated by p . Therefore the following equation results for $[na]$:

$$na = \pi n R^2 \delta - \pi n R^2 \frac{p}{s} \dots\dots\dots(7),$$

or, if $n = 1$,

$$a = R^2 \cdot \pi \left(\delta - \frac{p}{s} \right).$$

$\pi \left(\delta - \frac{p}{s} \right)$ represents a constant characteristic for the paper and may be indicated by k , so that

$$k = \pi \cdot \left(\delta - \frac{p}{s} \right)$$

and

$$a = R^2 \cdot k,$$

or

$$R = \sqrt{\frac{a}{k}} \dots \dots \dots (8).$$

That is to say, R depends only upon the variation in quality of the paper and upon the drop-volume which is taken, but it is independent of the concentration C . Therefore R is to be considered as constant for all concentrations, if the same paper and the same drop-volume are used.

The easiest and quickest way to find k is by means of the equation (8), but by weighing the paper and determining its specific gravity and thickness the same value for k will be obtained by the equation $k = \pi \cdot \left(\delta - \frac{p}{s} \right)$.

In order to determine the thickness δ of the paper, a certain number of sheets are placed between two glass plates and by pressing them very tightly the measure of the distance may be found, which gives when divided by the number of sheets a rough value of δ . The best manner to determine the specific gravities is the use of a pycnometer. The following data were thus obtained:

Paper No. 123	$\delta = 0.17$ mm. $p = 0.1408$ mgr. $s = 1.36$ $k = 0.20923$
Paper No. 117	$\delta = 0.16$ mm. $p = 0.118$ mgr. $s = 1.1$ $k = 0.1651$

When the value of k is found in this way, R can be calculated by the equation (8) as is shown in the following table:

TABLE VIII.

Paper No.	a (mgr.)	R calculated			R observed		
		a	$2a$	$3a$	a	$2a$	$3a$
123	116	23.537	33.28	40.77	23.48	32.09	37.9
117	116	26.49	37.48	45.90	26.5	35.75	42.28
123	13.1	12.165	—	—	12.8 Methyl-orange		
					14.6 Congo-red		

The agreement is very close for a , but here, too, it is apparent that increase of the number of drops increases the inexactness of the observations. If the value of R_1 is fixed, R_2 and R_3 can be calculated as $R_1 \sqrt{2}$ and $R_1 \sqrt{3}$, which give also a fairly good agreement, as the following figures demonstrate:

TABLE IX.

Paper No.	a (mgr.)	R_1	$R_2 = R_1 \sqrt{2}$	$R_3 = R_1 \sqrt{3}$
123	116	23.5	33.13	40.70
117	116	26.5	37.36	45.89

If $C = \frac{m}{a}$, the quantity na of HCl contains nm parts of it. These nm parts of HCl have been retained by adsorption in the inner circle (r), and if v indicates the volume of its interstices, the following equation must express the resulting concentration C_1 :

$$C_1 = \frac{n \cdot m}{v}.$$

I neglect the possibility, that the fibres may swell, supposing that the adsorption process is much sooner finished than the swelling of the fibres.

By analogy with equation (7), v can be expressed by

$$v = \pi n^2 \delta - \pi n^2 \cdot \frac{p}{s}.$$

After substituting this value for v , C_1 is given by

$$C_1 = \frac{n \cdot m}{n r^2 \pi \cdot \left(\delta - \frac{p}{s} \right)},$$

or

$$C_1 = \frac{m}{r^2 k} \dots \dots \dots (9).$$

This is the equation giving the resulting concentration in the coloured spot, and if z represents the increase of concentration, so that

$$C_1 = z + C,$$

and one substitutes

$$C_1 = \frac{m}{r^2 \cdot k}$$

and

$$C = \frac{m}{a},$$

the following equation results:

$$\frac{m}{r^2 k} = z + \frac{m}{a},$$

as

$$a = k \cdot R^2,$$

$$z = \frac{m}{r^2 k} - \frac{m}{R^2 k},$$

$$z = \frac{m R^2 - m r^2}{k \cdot R^2 \cdot r^2}.$$

If we substitute $k \cdot R^2 = a$,
 and $\frac{m}{a} = C$,
 we find $z = C \cdot \frac{R^2 - r^2}{r^2} \dots \dots \dots (10).$

Therefore z is found to be equal to the value of the paper-constant P of Holmgren (3).

Holmgren was of opinion that the relation between the resulting concentration C_1 in the paper and the original concentration C can be expressed by the quotient $\frac{R^2}{r^2}$, so that

$$C_1 = \frac{R^2 C}{r^2} \dots \dots \dots (11).$$

This view seems to be correct, for putting

$$C_1 = \frac{R^2 \cdot C}{r^2} = \frac{m}{r^2 k},$$

we find $R^2 \cdot C \cdot k = m$, and substituting $R^2 k = a$, the definition of $C = \frac{m}{a}$ results.

When Holmgren eliminated $r^2 = \frac{C \cdot R^2}{P + C}$ out of the equation (3) and substituted this value for r^2 in the equation (11), he got

$$C_1 = P + C \dots \dots \dots (12).$$

Therefore Holmgren's paper-constant corresponds with z in equation (10) representing the increase of concentration by the adsorption, P therefore not being a constant but a function of the concentration in question. The following table shows the values for C_1 calculated by the equation (9), P calculated by the equation (2) and C_1 calculated by formula (12).

TABLE X.

Paper No. 123. Congo-red. One drop ($a = 0.116$ gr.). $k = 0.20923$.

$C = \% \text{ HCl}$	$C_1 = \frac{m}{r^2 \cdot k}; \%$	$P = C \frac{R^2 - r^2}{r^2}$	$C_1 = P + C$	$P = 0.441$ $C_1 = 0.441 + C$
2.0	2.32	0.305	2.305	2.441
1.0	1.44	0.432	1.432	1.44
0.75	1.23	0.475	1.225	1.19
0.5	0.994	0.505	1.005	0.94
0.4	0.894	0.488	0.888	0.841
0.3	0.777	0.472	0.772	0.741
0.2	0.724	0.520	0.720	0.641
0.1	0.554	0.451	0.551	0.541
0.05	0.373	0.321	0.371	0.491

Mean of $P = 0.441$.

TABLE X (cont.)

Paper No. 117. Congo-red. One drop ($a=0.116$ gr.). $k=0.1651$.

$C = \text{‰ HCl}$	$C_1 = \frac{m}{r^2 \cdot k} (\text{‰})$	$P = C \frac{R^2 - r^2}{r^2}$	$C_1 = P + C$	$\begin{matrix} P=0.285 \\ C_1=0.285+C \end{matrix}$
1.0	1.19	0.194	1.194	1.285
0.75	1.05	0.299	1.049	1.035
0.5	0.825	0.325	0.825	0.785
0.4	0.668	0.286	0.686	0.685
0.3	0.593	0.292	0.592	0.585
0.2	0.524	0.323	0.523	0.485
0.1	0.377	0.277	0.377	0.385

We see that the values given by $C_1 = \frac{m}{r^2 k}$ and $C = P + C$ are almost identical as the theory demands. But if P is supposed to be constant according to Holmgren, and if the average value is taken for P , we find that the values no longer agree so closely. This difference is better demonstrated by the graphic method in constructing the C - C_1 -curve (Fig. 3).

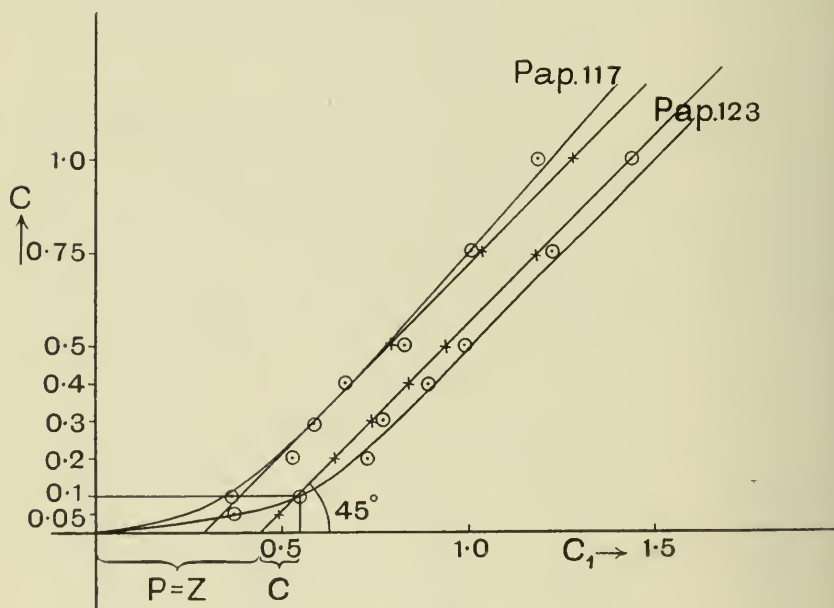


Fig. 3.

It is evident that $C_1 = P + C$ represents a straight line, which cuts the C_1 -axis at a distance P from the origin and the inclination of which must be 45° .

$C_1 = \frac{m}{r^2k}$ represents a parabola, by means of which it is possible to demonstrate the fact that for $C = 0$, C_1 must also be 0, a fact which is not expressible by $C_1 = P + C$, P supposed to be constant.

In reality, as P varies with the concentration, the equation $C_1 = P + C$ represents a system of parallel straight lines and the points indicating the relation between C and C_1 follow the course of a parabola cutting this system.

Nevertheless, the disagreement between Holmgren's supposition and the observed values is so slight, that for concentrations not under 0.1 % his method is for practical purposes very useful on account of its extraordinary facility. To demonstrate the very close agreement of the calculation by Holmgren's formula (2) with the reality I give the following table:

TABLE XI.

Paper 123. $a = 0.116$ gr.

$C = \% \text{ HCl}$	$P = 0.441 \text{ (const.)}$	$m = \frac{0.273}{\sqrt{18.88}} \frac{r}{r^2}$
	$C = P \frac{r^2}{R^2 - r^2}$	$\frac{m}{a} = \%$
2.0	2.87	1.4
1.0	1.02	0.989
0.75	0.69	0.77
0.5	0.43	0.53
0.4	0.36	0.44
0.3	0.28	0.33
0.2	0.16	0.18
0.1	0.097	0.084
0.05	0.068	0.048

With regard to a possible combination of the dye and the acid in the paper, the amount of Congo-red or methyl-orange can be approximately calculated. Bayliss [1906] has shown in regard to Congo-red, that the staining of paper is an adsorption process. The temperature-coefficient of the reaction-velocity is so low, that at room temperature at least 24 hours are required for the attainment of equilibrium. I used a clear looking alcoholic solution of Congo-red (0.04 %) and of methyl-orange (0.1 %) always at room-temperature, and dipped the piece of paper into these solutions only for a few seconds, then allowing the alcohol to drain off and drying the paper at 37°. It can therefore be presumed that the amount of dye adsorbed is very small, and if it be assumed that in so short a time only that amount of Congo-red (or methyl-orange) can be adsorbed which was present in the solution filling the interstices of the paper-fibres, the possible error is certainly very small.

Of an 0.04 % alcoholic Congo-red solution each cmm. contains 4.10^{-7} g. dye,

or in case of 0.1 % methyl-orange $0.1 \cdot 10^{-7}$ g. dye. As already shown, the volume of the interstices, which belongs to a surface of πr^2 , has been found to be $r^2 \cdot k$. According to Table I r was found = 10 mm. in case of an 0.1 % HCl, which gives $r^2 \cdot k = 20.9$ or 21 cmm. Therefore the amount of dye adsorbed in these 21 cmm. is in case of Congo-red $84 \cdot 10^{-7}$, and in case of methyl-orange $2.1 \cdot 10^{-7}$. The same consideration gives $2.1 \cdot 10^{-7}$ as the amount of an 0.1 % HCl in the same volume of interstices.

These figures are mentioned although I was not able to prove that any chemical action resulting in a perceptible decrease in the amount of free acid took place between dye and acid. No difference in the measurement of the radii of the acid-circles could be observed on varying the concentration of the indicator-solution. Holmgren, who compared the effect of 1.0 and 0.1 % solutions of Congo-red in water on the extent of the surface produced by allowing a drop of acid to fall on paper under similar conditions, thought it also very improbable that the amount of dye by itself plays any important part.

But if the effect of adsorption in unstained paper is compared with that obtained in stained paper, a difference can be observed.

Experimenting under the conditions described in the introduction, one observes in unstained paper that the extent of the water-zone exceeds by 2.5 mm. that found in paper stained by the alcoholic indicator-solutions. This difference in the extent of the water-zone is constant, when the concentration of the acid employed varies, a fact which agrees with the circumstance that R is independent of the concentration.

The extent (r) of the acid-spot is also less in stained paper than in unstained, but according to observation this difference increases the more the acid has been diluted, which favours the idea that a chemical action occurs between acid and indicator.

The weight of about 500 gr., which I used to prevent the paper from wrinkling, influences the extent of the water-zone, but the enlargement caused by the pressure is very insignificant and does not exceed 0.1 mm.

In studying the influence of pure alcohol on the paper regarding the adsorption of hydrochloric acid and the capillary extension of water, experiments showed that pure alcohol has an inhibiting action on water as well as on diluted acid.

I may suggest that the fibres of the paper when treated by alcohol, whether pure or combined with dye, are inhibited or prevented from swelling by water, and thus the interstices become larger, which would explain the observation that the radii become smaller.

To apply these considerations to the method which involves the measurement of the capillary height in strips of paper the different volume of liquid must be taken into account. Skraup and his co-workers [1910] were able to show that the amount of water raised in strips of filter-paper is different in different parts of the strip, decreasing in a hyperbolic manner [p. 887]. Whether a similar decrease of the amount of water occurs in the case of a drop producing a circle on the filter-paper is not proved, and I think it very improbable, believing that this phenomenon depends upon gravitation and therefore upon the inclination of the strip, the influence of which is shown by Goppelsroeder [1909] in regard to the capillary height.

I found also in strips of paper that the fact whether they are untreated, stained by alcoholic Congo-red solutions or only treated by pure alcohol, must be taken in account. Thus under the same experimental conditions distilled water rises quicker in the unstained paper and the contrary takes place in case of a dilute hydrochloric acid.

The following table contains measurements of capillary heights in mm., the time having been constant for each experiment, but I could not find any satisfactory explanation of the phenomenon.

		Paper 123 untreated	Treated by	
			Pure alcohol	Congo-red
Diluted HCl	{ Water	59	71	71
	{ Acid	50	59	59
Distilled water	{	85	105	77
		66	78	62
		76	89	69

Holmgren [1908] found that the relation between the capillary heights of the acid and the water increases according to the concentration, and that this relation is constant for the same concentration; Skraup and his co-workers [1910] found for all acids (with a few exceptions) that the stronger the acid the higher is the degree of adsorption and *vice versa*.

I hope later to be able to show how the mathematical considerations described in this paper can be applied also to Goppelsroeder's and Skraup's experimental data obtained by the measurement of the capillary height. But I am conscious of the fact that these formulae are still far from being able to describe all the possibilities in such a complex phenomenon as the adsorption of acids by paper.

CONCLUSIONS.

1. Diluted acids produce a ring system when dropped on blotting-paper, the acid remaining behind the water.

2. The radius r of the coloured circle produced by the acid in the paper is connected with the concentration C by an exponential equation of the form $r = \beta \cdot C^n$.

3. The radius R of the water-zone is independent of the concentration and can be determined by the equation $R = \sqrt{\frac{a}{k}}$, a being the volume of the acid drop in question and k being a constant which depends upon the quality of the [stained] paper.

4. The adsorption causes an increase of concentration. The final concentration is found to be a parabolic function of r .

5. The increment of concentration varies with the initial concentration.

6. Holmgren's calculation, which assumes that the increment of concentration is a constant dependent only upon the quality of the paper, is theoretically incorrect, but it has been shown that it may be useful for practical purposes.

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XXXVII. THE BIOCHEMICAL SYNTHESIS OF THE FATTY ACIDS.

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(Received June 10th, 1913.)

The methods by which fat is formed in the living organism remain at present completely unknown to us. Even the chemical reactions by which fatty acids are built up, comparatively simple though these must be, have hitherto found no satisfactory explanation. The one fact which appears to be completely established, by a large mass of experimental evidence, is that the carbohydrate of the food may be converted into fat inside the living organism, although neither the place where this change takes place nor the method by which it is accomplished is known with any degree of certainty.

The evidence as to the formation of fat from protein is less convincing, but it is at any rate possible that a conversion of protein into fat may also take place.

But little may be learnt from an attempt to correlate the composition of the fat stored up in the organism with the nature of the food supplied. There is abundant evidence that fatty acids taken in the food may be merely stored up unchanged in the body. The glycerides of palmitic, stearic and oleic acids are the constituents which most generally occur. Acids belonging to more highly unsaturated series than oleic acid have been demonstrated but these are more probably connected with further changes in the building up of fat into complex molecules, possibly of the nature of lecithin, than with the synthesis of the fatty acids themselves.

Two of the most prolific factories of fat are perhaps to be found (1) in plants, in such nuts as that of the cocoa-nut tree (*Cocos Nucifera*), where an abundant transformation of carbohydrate into fat must take place, and (2) in animals in the active mammary gland.

In both these instances, where a comparatively rapid conversion of

carbohydrate into fat is probably taking place the resultant fats are characterised by the presence of considerable quantities of the lower fatty acids. In cocoa-nut oil, the acids containing the even numbers of carbon atoms from six to eighteen, in butter from four to twenty, have been described. In these acids the carbon atoms are linked in straight chains and there is no evidence that any acid with a branched structure exists.

The question now arises whether the normal fatty acids present in butter are products of synthesis or of degradation. Knoop [1904] and Dakin [1908, 1909] have shown that the fatty acids are broken down by oxidation of the β -carbon atom; all the lower fatty acids present in butter may therefore be derived by oxidation from the arachidic or stearic acids present.

Some evidence on this point may be obtained from agricultural experiments; the problem has been directly investigated in an attempt to determine the reason of the variations which occur in the proportion of volatile fatty acids present in butter fat. Swaving (1906) carried out feeding experiments in the North of Holland to determine the cause of the low percentage of volatile soluble acids. Van der Zande and Siegfeld showed that a diet rich in carbohydrate, e.g. turnips, increased the proportion of the lower fatty acids and more recently Siegfeld [1907] and Amberger [1907] have shown that the increase is more especially in the insoluble volatile acids (i.e. caprylic, capric and lauric). Amberger, in a series of experiments carried out on the same set of cows showed that whereas food rich in protein such as malt germs diminishes the proportion of lower fatty acids, food rich in carbohydrate such as turnips increases this proportion. If the percentage of the lower fatty acids increases with the amount of the carbohydrate in the food, it would appear more probable that they exist as intermediate synthetic products on their way to the higher fatty acids, than as degradation products. Such evidence as exists is therefore in favour of a synthesis in which all fatty acids containing even numbers of carbon atoms from four to twenty linked together in straight chains are formed from carbohydrate in some way through the agency of the mammary gland.

Previous Hypotheses as to the Nature of the Reactions by which Fatty Acids are formed from Carbohydrate in the Animal Organism.

Emil Fischer suggested that stearic and oleic acids are formed by the condensation of three hexose molecules or of six triose (glycerose) molecules in such a way that a straight chain containing 18 carbon atoms

is formed. From this, by further processes of oxidation and reduction, stearic and oleic acids are formed. Palmitic acid with its chain of 16 carbon atoms would be compounded from two pentose and one hexose molecules. Glucose, gluconic and glucuronic acids are suggested as the precursors of the pentose molecules. In favour of this hypothesis it is difficult to find any evidence either of a chemical or biological nature. Against it the following considerations may be urged:

(a) No laboratory method is known by which two hexose molecules may be made to condense in such a way that a straight chain of twelve carbon atoms is produced.

(b) Pentoses are known to exist in the organism in combination in the nucleoproteins but there is no indication that the pentoses are in any way connected with the formation of fat or with normal carbohydrate metabolism.

(c) If it be granted that the fatty acids of butter are products formed synthetically from carbohydrate, the hypothesis presents insuperable difficulties. No combination of hexose and pentose molecules will produce myristic acid ($C_{14}H_{28}O_2$) by direct addition, yet this acid occurs commonly in fats, e.g. lard, butter, cod-liver oil and many vegetable fats. The existence of an intermediate tetrose sugar would have to be assumed as a normal constituent.

3 hexose molecules	give stearic acid.
2 pentose and one hexose molecules	„ palmitic acid.
2 pentose and one tetrose	„ myristic acid.
2 hexose molecules	„ lauric acid.
2 pentose „	„ capric acid.
2 tetrose „	„ caprylic acid.

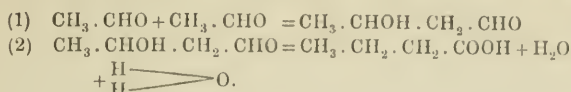
There is no evidence of the formation of a tetrose molecule in the organism and it is exceedingly unlikely that a regular series of fatty acids should be formed in this way.

(d) In the case of stearic acid the reduction of seventeen hydroxyl groups would be assumed.

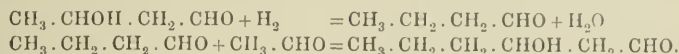
This hypothesis has therefore no evidence in its favour and involves reactions which are not analogous with any of those known to us. It does not therefore furnish us with a satisfactory explanation of the problem under consideration.

The second hypothesis, which is perhaps the more generally accepted, is that the fatty acids are built up by repeated condensations of a compound containing two carbon atoms. This was first suggested by Nencki and afterwards developed by Magnus-Levy [1902], Leathes and others who regarded acetaldehyde as the substance from which by a series of aldol condensations

the straight chains containing even numbers of carbon atoms were formed. The reactions involved would be represented by the following equations:



If the aldol on the other hand were reduced to butyl aldehyde, it would be available again to take part in a similar condensation:



a normal six-carbon-atom chain being thus produced.

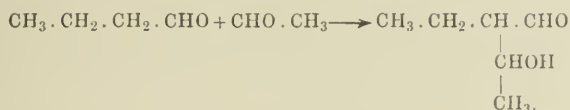
In favour of this hypothesis, it may be urged:

(a) It does account for the production only of those fatty acids containing even numbers of carbon atoms, since only multiples of two will exist.

(b) Hoppe Seyler showed that by the action of caustic alkali on lactic acid at from 200° – 300° , acetic, butyric and caproic acids were formed. Pasteur had previously shown that butyric and caproic acids were formed by the bacterial fermentation of sugar. Acetic aldehyde may be obtained from lactic acid and may therefore be a degradation product of sugar.

On the other hand it is open to the following criticisms:

(a) Lieben [1883, 1901] and his pupils have shown that when the higher aldehydes condense with acetaldehyde under the influence of dilute alkalies, the resulting aldehydes possess a branched and not an open-chain structure:



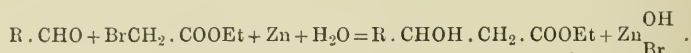
It has since been shown that both aldol and crotonaldehyde will undergo auto-condensation with the formation of a normal eight-carbon-atom chain [Raper, 1907; Smedley, 1911]; but the difficulty of adding on acetic aldehyde to a higher aldehyde so as to build up chains increasing by the addition of two carbon atoms has not been surmounted. One must therefore assume that the condensation of aldehydes in the body does not take place in the same manner as it does when brought about by the action of condensing agents in the laboratory.

(b) No free aldehydes other than the sugars have been detected in the body. If present in quantity they would probably be injurious to the life of the cell. Parnas [1910] has shown that an enzyme is present in the liver by which free aldehydes are at once removed.

(c) There is no biological evidence that acetaldehyde is formed as an intermediate substance in the body metabolism.

The aldol condensation does not therefore furnish us with a satisfactory analogy for the method by which the fatty acids are built up.

A survey of the general methods of producing fatty acids in the laboratory shows that the most satisfactory method by which fatty acids may be built up by increments of two carbon atoms is by means of Reformatski's reaction in which aldehydes are condensed with bromoacetic ester in the presence of zinc;

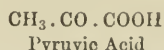


As however neither zinc nor bromoacetic ester occurs in the body, this does not furnish us with any helpful analogy for biochemical synthesis.

The Degradation-products of Carbohydrates: their suitability as Units in the biochemical synthesis of Fatty Acids.

But little is known as to the manner in which carbohydrate breaks down within the body. It has been repeatedly established that when a solution of glucose in Ringer's fluid is perfused through the isolated heart sugar disappears [Locke and Rosenheim, 1907; MacLean and Smedley, 1913]. This is the only instance in which it has been established beyond the region of controversy that sugar disappears when subjected to the action of an isolated organ. But even here the decomposition products of the sugar molecules are unknown. The controversy as to whether glycogen is a storage product or a stage in the normal metabolism of sugar throws little light on the problem under consideration. The discussion as to whether glucose is the source of the lactic acid in the animal organism has more bearing on the subject of fat formation. Embden has shown that the transfusion of blood rich in sugar through a glycogen-free liver resulted in the abundant formation of lactic acid: blood poor in sugar similarly transfused gave rise to lactic acid in inconsiderable amount. The formation of lactic acid from carbohydrate is also indicated by the experiments of Mandel and Lusk on phlorizin diabetes. It seems probable that both carbohydrate and protein may give rise to lactic acid in the body. The occurrence of lactic acid as a possible cleavage product of carbohydrate suggests that the breaking down of sugar takes place in such a way as to give rise to compounds containing three carbon atoms. Lactic acid itself is not a very reactive substance nor does

it appear a hopeful starting material for the synthesis of fatty acids. It is however closely related to pyruvic acid.



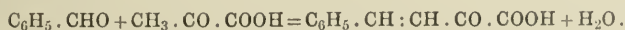
There is also evidence that pyruvic acid itself is probably of considerable importance in animal metabolism. It has been demonstrated that a close connection exists in the organism between the α -amino- and the α -keto-acids. Embden and Schmitz [1910, 1912] have shown that if a solution of ammonium pyruvate be perfused through a liver, alanine is formed. Fellner [1912] further showed that if a liver rich in glycogen be perfused with blood containing ammonia, alanine is formed, and from a consideration of Embden's experiments pyruvic acid is indicated as the intermediate substance. Neubauer and Knoop and Kertess [1911] have also suggested the formation of alanine from pyruvic acid in the body.

Knoop [1910] and Knoop and Kertess [1911] have shown that if γ -phenyl- α -amino-butyric acid be fed to a dog, a considerable proportion of the acid appears in the urine as the acetyl derivative; the same phenomenon was observed by Neubauer and Warburg [1910] in their perfusion experiments. There is some reason to believe that the acetylating agent may be pyruvic acid, since de Jong [1900, 1904] showed that ammonium carbonate and pyruvic acid react with formation of acetyl-alanine. It seems therefore probable that pyruvic acid may be an intermediate substance formed in the body from carbohydrate.

Pyruvic acid is a reactive substance, readily losing carbon dioxide under the influence of oxidising agents and forming acetic acid. A study therefore of its chemical properties and of its powers of condensation seemed of especial interest.

The condensation of Pyruvic Acid with Fatty Aldehydes and the oxidation of the products formed.

It had already been shown that if anhydrous hydrochloric acid be passed into a mixture of benzaldehyde and pyruvic acid, cinnamoyl-formic acid results [Erlenmeyer, 1901];



Later both benzaldehyde and cinnamyl aldehyde were condensed with pyruvic acid by adding a small amount of 10% caustic soda to the mixture [Erlenmeyer, 1903].

In order to make use as far as possible only of reagents which may be considered to bring about reactions somewhat similar to those brought about

by enzymes within the body, the condensation of the fatty aldehydes with pyruvic acid was attempted in very dilute alkaline solution. The intermediate unsaturated α -keto acid which was expected to result was not isolated, but the product was at once oxidised by silver oxide in alkaline solution or by hydrogen peroxide in neutral solution.

The behaviour of crotonaldehyde was first investigated.

EXPERIMENTAL.

Condensation of crotonaldehyde with pyruvic acid and oxidation of the product formed.

5 grams pyruvic acid, 5 grams crotonaldehyde, 75 cc. n. NaOH and 1 litre of water were added together and left for three days at the room temperature, the solution being approximately 1/50 normal. The liquid became deep yellow but no insoluble oil separated as in the condensation of crotonaldehyde alone. The solution was neutralised by the addition of 12.5 cc. n. H_2SO_4 and steam distilled to remove any free aldehyde.

Oxidation of Reaction-product.

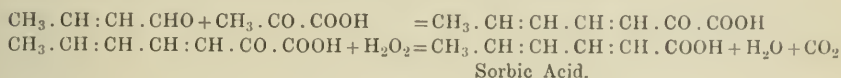
Silver oxide was precipitated from 30 grams silver nitrate and added to the solution of the condensation product of crotonaldehyde with pyruvic acid after it had been steam distilled. 200 cc. of a 1/3 normal solution of baryta were gradually added and the whole allowed to stand over night. Next morning the silver oxide had been largely converted to silver. The precipitate was filtered off, washed and concentrated under reduced pressure until 50 cc. remained. In order to convert any hydroxy-acid that might conceivably be present to the corresponding unsaturated acid, 10 grams of baryta were added and the mixture boiled for 30 minutes.

Excess of sulphuric acid was then added and the whole steam-distilled. 1500 cc. of the steam distillate required 28.5 cc. normal potash for neutralisation. The neutral distillate was evaporated almost to dryness and to the potassium salt so obtained, 10 % H_2SO_4 was added. Crystals separated which melted at 132° after once recrystallising from dilute alcohol. The melting point was unchanged on mixing with a specimen of sorbic acid prepared by the condensation of crotonaldehyde and bromoacetic ester, and hydrolysis of the ester formed. The crystals were therefore satisfactorily identified as sorbic acid.

In subsequent experiments the oxidation of the neutral condensation product was carried out by means of hydrogen peroxide. An amount of hydrogen peroxide exactly equivalent to the pyruvic acid originally taken

was used and the neutral mixture of condensation product and peroxide left to stand over night at the ordinary temperature; the product was concentrated under reduced pressure and steam distilled as before, and the final product consisted of a mixture of acetic and sorbic acids, the yield being somewhat improved by this means. From 5 grams of crotonaldehyde 0.5 g. sorbic acid was thus obtained.

The reaction must therefore have proceeded as follows:



The condensation of Butyl Aldehyde with Pyruvic Acid.

10 grams of butyl aldehyde, 10 grams of pyruvic acid and 150 cc. normal potash were shaken up with 2 litres of water and at the end of 12 days the mixture was neutralised and concentrated under diminished pressure. To the concentrated residue silver oxide from 43 grams of silver nitrate and 200 cc. n/3 baryta were added. After standing over night, the silver precipitate was filtered off and the filtrate concentrated to 250 cc., 50 grams of baryta added and boiled for 30 minutes. The whole was then acidified with dilute sulphuric acid and distilled in steam. 1500 cc. of distillate were neutralised by 59.2 cc. normal potash and evaporated to dryness. The residue was acidified and extracted with ether. After evaporating off the ether, the residue was distilled under a pressure of 20 mm. About 3 grams boiling from 130°–140° were obtained. The liquid rapidly decolourised bromine water and gave on analysis the numbers required for the compound $\text{C}_6\text{H}_{10}\text{O}_2$.

0.1409 g.; 0.3262 g. CO_2 ; 0.1134 g. H_2O .

“ C 63.14 % H 8.94 %.

Calc. for $\text{C}_6\text{H}_{10}\text{O}_2$ C 63.15 % H 8.77 %.

In subsequent experiments, the product similarly prepared appeared to consist of a mixture of octylenic acid (probably obtained by the self-condensation of the butyl aldehyde) and of hexylenic acid obtained from butyl aldehyde and pyruvic acid. The difficulty of separating these in a small quantity of a liquid mixture is considerable.

In another experiment where hydrogen peroxide was used as the oxidising agent as described under crotonaldehyde, the product obtained distilled under reduced pressure (15 to 20 mm.) from 120°–128° and gave on analysis the following numbers.

0.1220 g.; 0.2858 g. CO_2 ; 0.1074 g. H_2O .

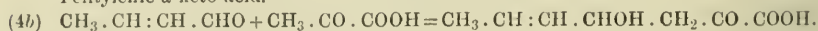
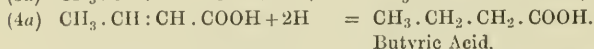
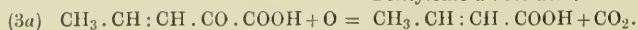
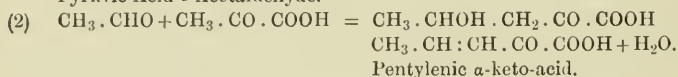
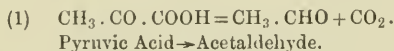
C 63.85 % H 9.75 %.

In investigating the condensation of iso-valeraldehyde and oenanthol with pyruvic acid, chiefly the products of condensation of the aldehydes with themselves were isolated. From the condensation product of iso-valeraldehyde and pyruvic acid, a small amount of the barium salt of an acid was obtained, the percentage of barium in which agreed with that required for the barium salt of the corresponding unsaturated keto-acid.

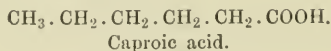
Condensation of these higher fatty aldehydes with pyruvic acid under varying conditions is now being investigated. The condensation of croton and butyl aldehydes with pyruvic acid and the oxidation of the product formed with hydrogen peroxide in neutral solution furnishes a method by which an unsaturated fatty acid may be built up containing two more carbon atoms than the aldehyde from which it is derived. These condensations have also been investigated under similar conditions in the aromatic series [Smedley and Lubrzyńska, 1913].

CONCLUSIONS.

The hypothesis now brought forward [Smedley, 1912] suggests that pyruvic acid, formed in the body as a decomposition product of carbohydrate, is the starting-point for the synthesis of the fatty acids. The stages which are assumed to occur are represented by the following equations:



and by reactions similar to 3a and 4a



The evidence supporting this hypothesis may be briefly summarised as follows.

1. Pyruvic acid is probably a degradation product of carbohydrate in the body.

The perfusion experiments of Embden, Knoop and Neubauer show that pyruvic acid is converted into alanine through the agency of the liver cells

and that a close connection exists between the α -amino- and α -keto-acids. Pyruvic acid may probably be an intermediate stage in the transformation from glycogen to alanine (Fellner).

There is some reason to believe that in the acetylation of certain amino-acids which has been observed both in perfusion and in feeding experiments, pyruvic acid is the acetylating agent.

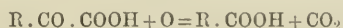
The close connection between alanine and pyruvic acid suggests that the alanine group of the protein molecule may furnish an additional source of the pyruvic acid available for the synthesis of fatty acids.

2. The decomposition of pyruvic acid into acetaldehyde and carbonic acid, which constitutes the first stage of this process, has been shown by Neuberg to be readily brought about by an enzyme present in yeast, termed "carboxylase."

3. The present hypothesis postulates that free acetaldehyde is not liberated but that the decomposition of the keto-acid is in some way regulated by the pyruvic acid with which the "nascent" aldehyde combines.

The condensation of fatty aldehydes with pyruvic acid has now been shown to take place in the laboratory under the influence of dilute alkalis at ordinary temperature.

4. Oxidation of the α -keto acid according to the equation



may be brought about in the laboratory by hydrogen peroxide at the ordinary temperature in neutral solution (p. 370).

5. The reduction of the unsaturated acid is the final stage; there is abundant evidence that reduction can take place in the body although very little is known as to the mechanism by which it is accomplished.

6. The α -keto-acid, synthesised as above, may be split into CO_2 and aldehyde, and a further condensation with pyruvic acid may then be effected. An acid with two more carbon atoms than the original aldehyde would thus be synthesised.

As yet no α -keto-acids have been detected within the body: it may be that they occur only within the cell and that reduction or oxidation always accompanies their liberation. The above hypothesis accounts for the formation of a series of straight chain acids beginning with four carbon atoms and increasing by increments of two carbon atoms: it involves only reactions which are analogous with those which are known to occur in the laboratory and there is reasonable evidence for believing that the starting material, pyruvic acid, can be formed from carbohydrate in the body.

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“*Crithidia*” fasciculata in hibernating mosquitoes (*Culex pipiens*)
and the question of the connection of this parasite with a Trypanosome.

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(With 41 figures.)

In the course of my work on the blood parasites of birds and the manner of their transmission, I have been able, recently, to give some attention to the above subject. Towards the end of September, 1912, Mr. Bacot, the Entomologist at the Lister Institute, brought me some female individuals of *Culex pipiens* from the cellar of his house; the Insects had entered upon their period of hibernation in the cellar, which is dark and relatively humid. No male individuals occurred in this situation. I take this opportunity of thanking Mr. Bacot warmly for his kind assistance in supplying me with both adult Insects and larvae on numerous occasions, and for his helpful advice and suggestions.

On examining one of these females, I was greatly surprised to find the intestine crammed with Flagellates, the great majority of which were undoubtedly the much-discussed "*Crithidia*" *fasciculata*, of Léger. I examined more of the *Culex* to see whether the infection was common and found that nearly half (i. e. 4 out of 9) were infected. So far as I know, this is the first occasion on which the occurrence of Flagellates in hibernating *Culex* has been noted since the publication of Schaudinn's celebrated work. The only reference bearing on the subject of

which I am aware is a brief mention by Léger and Duboscq¹ of the presence of Crithidia in a hibernating *Anopheles*. The earliest notice of Flagellates in *Culex* is the graphic description of Ronald Ross², with which the general condition of the parasites and their behaviour under observation, in the present case, agree very well, leaving aside for the moment the question of the exact nature of the forms seen by Ross. The vast majority of the Flagellates were in the resting, attached phase; they formed a carpet along the surface of the wall of the intestine, with their flagellar ends in contact with this, and also constituted large "rosette" rosettes, blocking the lumen to a very considerable extent, and sometimes causing distension of the wall. In one or two mosquitoes, the digestive tracts of which were immediately looked at whole, without having been ruptured at all, a few free, actively moving Flagellates were seen, about the pyloric region of the stomach. Nearly all the resting forms had the typical appearance of a barley grain, as it was first characterized by Léger³. In most, a very short free flagellum was present, little more than a spike-like projection (figs. 4, 18), but some individuals had practically no free flagellum. In such, however, the attached, or "fixed" portion of the flagellum was always present, and is clearly seen in the stained preparations (figs. 1—3).

A remarkable feature of the parasites which was noticed by Ross and the significance of which has not been pointed out by more recent workers, is their behaviour when brought into contact with water. As soon as the infected part of the digestive tract was teased up, either in ordinary water or in salt-solution, and the liquid had access to the flagellates, these became active; many of them broke away immediately from the cluster of which they formed part and swam about vigorously, others following suit after a short interval. This resumption of the active condition took place in nearly all the individuals which came into contact with the water, only a small proportion of those hemmed in, as it were, in the middle of a large clump remaining motionless. In the great majority, the flagellum must have developed almost at once to its full length. This process appeared to take place just as much in the parasites situated in the rectal part of the digestive tract as in those in other parts of the intestine, numbers of active individuals swimming out of the cut, anal end of the rectum, behind the glands. No encysted forms were observed in the living preparations studied (but cf. below).

While most of the active parasites were fairly short, a few elongated, more herpetomonad-like ones were seen. In neither form, however,

¹ C. R. Ass. franc. avanc. sci., 31. 1902. p. 703.

² Vide his resumé in J. Hyg. 6. 1906. p. 101.

³ C. R. soc. biol. 54. 1902. p. 354.

could anything like an undulating membrane be made out; nor did the movement of the parasites suggest the presence of one, the body being held rigid and progression being in a straight or slightly zig-zag course, produced entirely by the vibrations of the flagellum.

In the stained preparations, the shape of the body and the general morphology of the short forms agree entirely with the original descrip-

Fig. 1—6.



Fig. 7—12.



Fig. 13—17.



Fig. A. (All figs. are magnified about 2500 times linear.) All the parasites are from mosquitoes at the commencement of the hibernating period (autumn). 1—3, typical resting forms; 4, individual with very short spike-like flagellum; 5—14, active individuals with the typical truncated or oval shape (*grain d'orge*); 10, a parasite undergoing division; 15, rather larger, stouter form; 16 and 17, herpetomonad (semi-herpetomonad) forms. Note the conspicuous granules in many of the parasites.

tion of Léger, and with the account and microphotographs of this parasite given subsequently by Novy, Mc-Neal and Torrey⁴. The

⁴ J. infect. diseases, vol. 4. 1907. p. 223, many pls.

two nuclei are usually very close together, situated often in the posterior half of the body; the kinetonucleus is generally alongside of (opposite to) the trophonucleus, but it may be slightly in front of, or even behind the latter [cf. figs. 5, 18, 21, 23]. As a result of this, the attached

Fig. 18—22.



Fig. 23—28.



Fig. 29—33.



Fig. B. (All figs. are magnified about 2500 times linear.) All the parasites are from mosquitoes after hibernation (in spring). 18, form with short, spike-like flagellum; 23—26, typical "crithidial" individuals; 27 and 28, transitional forms between the latter and, 29—33, herpetomonad or semi-herpetomonad parasites. Note the general absence of conspicuous grains in the cytoplasm.

tion of the flagellum is frequently of considerable length; even in such cases, however, it appears to be closely attached to the side of the body and I cannot note any indication of a membrane. In some indivi-

duals, the anterior end of the body, instead of being abruptly truncated (figs. 9—13, 21), at its anterior end, tapers gradually, being drawn out, as it were, along with the flagellum (figs. 25, 26, 28); one can hardly call this, however, a rudimentary membrane. In the longer individuals, the tendency is to a more herpetomonad condition; the kinetonucleus is always well in front of the trophonucleus and may sometimes be near the anterior end (figs. 16, 17, 29—31), the trophonucleus remaining, however, near the middle of the body. The anterior end usually thins out along the flagellum and in this respect differs from the blunt extremity characteristic of a typical *Herpetomonas*. These forms recall, I may note, certain cultural phases of an Avian Trypanosome which I have described⁵, though I do not wish to lay any stress upon the point. Although I have not found these elongated forms in all the preparations made of infected parasites, I think there can be no doubt that they belong in the same life-cycle as the short "crithidial" forms. A regular series of transitional phases between the two can be readily found (cf. figs. 14, 27—29).

Many of the fixed and stained parasites shew a cytological peculiarity which probably stands in some relation to the rapid development of the free flagellum. In individuals caught in the resting phase, which have no free flagellum, there is usually a conspicuous pink-staining area or patch at the anterior end of the body (figs. 1—3); this does not appear to me to be a vacuole. The attached, or body-portion of the flagellum, which is always well-defined in resting (non-encysted) forms, runs from the neighbourhood of the kinetonucleus to this area, in which it seems to merge. The same pink-staining substance is frequently present also in individuals which have a short, spike-like flagellum, but its area is much less (cf. figs. 5, 18). In the forms with fully developed flagellum there is rarely any sign of it. Apparently, this area represents the substance of the retracted, free portion of the flagellum.⁶

In the preparations made in the autumn, many of the parasites, both short and elongated forms, contain numerous large, deeply staining granules, situated chiefly in the posterior region of the cytoplasm. These probably represent reserve food-material.⁷

I will mention here that in one of the preparations made of the four infected digestive tracts in the autumn, a few cysts have been found after much searching, but these will be more suitably described after considering the infection in the mosquitoes in the subsequent spring, when the cysts were first observed.

With a view to seeing whether the Flagellates would persist alive

⁵ Vide Quart. J. Micr. Sci. vol. 55. 1910. pl. 30, figs. 140, 141.

and in the same condition in the infected mosquitoes throughout the winter, I refrained from examining more in the autumn, but endeavoured to keep a number alive, which Mr. Bacot brought me. The mosquitoes were placed, some in small cardboard boxes, and the rest in stiff paper rolls, loosely plugged at both ends with cotton-wool. Both lots were covered over with fine sand, to retain dampness. Some of both lots were placed in a fairly damp spot in a cool greenhouse, others were kept in a room, cold but comparatively dry. Half-a-dozen or so of the mosquitoes were left to their own devices in an ordinary mosquito cage in the same room. These last were observed at intervals during the course of a month, remaining motionless during this time. At the end of October one of them was examined and just the same condition of affairs was found, the parasites being abundant. All were then left undisturbed over the winter, and at the end of March I looked to see how they had fared. Three were found still alive in the cage, but unfortunately not one of the others, specially cared for, remained alive. They had all been destroyed by a blue mould. This was entirely unexpected, both by Mr. Bacot and myself. It was certainly neither the effect of temperature nor of the degree of humidity, for, as just noted, some were still alive in my (relatively) dry room and Mr. Bacot found also some still in his (relatively) very damp cellar, although here, too, the mortality had been very high. In the case of those I kept, it was probably the confining of the air, even to the extent caused by the loose cotton-wool, which had sufficed to kill them.

In one of the three which remained alive in my cage and in about 50% of those examined which Mr. Bacot was again able to obtain from his cellar in the spring, the parasites were present, and broadly speaking, in the same condition as they were in at the beginning of the hibernation, both as regards numbers, form and behaviour. One point of difference, which is well seen in the permanent preparations, is that the parasites now lack the large conspicuous granules which occur in many in the autumn preparations. At the most, a few fine granules are observable in some individuals (figs. 24, 29). For this reason, it is probable that the large granules represent reserve food material which has been used up by the parasites during the winter. During the whole of this period, of course, the intestine of the mosquito is empty.

The most surprising feature about the parasites is that after this long quiescent period, they practically all become just as active when the alimentary canal is broken up, as those did which were present in the females examined in the autumn. I did not at this time see any cysts in the living examinations, any more than I did in the autumn. When I found that the parasites, although motionless, were merely in the same

resting condition and swarmed out actively from any part of the ruptured intestine or rectum just as before, on the liquid reaching them, I did not expect to find any encysted forms. It was naturally to be expected that, if the Flagellates were going to encyst, the great majority would be encysted after that lapse of time, especially in the hinderpart of the digestive tract. As a matter of fact, however, in studying the permanent preparations made in the spring, of these infected digestive tracts, with a view to making drawings for this paper, I have recently come across a few encysted forms from one gut, as well as a certain number of parasites commencing to encyst. Nevertheless, in view of the enormous number of individuals actually present, the encystment is taking place to a surprisingly small extent, and it is no wonder that the few cysts were not observed in life. Moreover, in two other cases, preparations have been thoroughly searched with out a single cyst being found. On the other hand, in one of the 4 infected digestive tracts preserved in the autumn, renewed search has shewn the presence also of a few cysts. The important point therefore, is that both at the end of the period of hibernation, as at the commencement, the vast majority of the parasites are unencysted.

The cysts of "*Crithidia*" *fasciculata*, which are described here for the first time, so far as I am aware, are very similar to those of other Flagellates occurring in Insects, which are already known. They are oval or slightly pear-shaped (figs. 37, 39), in the latter case, the hinder end of the body forms the broader end of the pear. They vary somewhat in size, being usually rather larger than the small truncated, "crithidial" parasites, and are apparently formed by intermediate-sized individuals (cf. figs. 4, 15), or by semi-herpetomonad forms (fig. 30). The cyst-wall is fairly thick, especially at the posterior end, where it is often much thicker than at other parts. It stains deeply with Giemsa and its external contour is at times rather irregular (fig. 40); it is most probably formed of the same kind of semi-granular, semi-viscid secretion which constitutes the wall of the "Schleimeysten" of Prowazek. In some cases, the wall stains so intensely that the cyst appears very dark and opaque (fig. 41). The body of an encysted parasite is often very granular, now and again rendering it difficult to distinguish the limit of the trophonuclear area (fig. 40). The kinetonucleus, which is always quite definite, is situated close to the trophonucleus. Sometimes the basal part of the attached flagellum (rhizoplast?) can be made out in the completed cyst (figs. 38, 41), but in other cases, it is no longer distinguishable (figs. 39, 40). Stages in cyst-formation are seen in figs. 34-37. The outline of the body becomes less sharply defined, with fine granules adhering to it, which perhaps represent the commencing secretion

g. 34]. In some individuals the cyst-wall is secreted first at the posterior end and attains its full thickness there before it is completed around the body (figs. 36, 37); but in other cases it appears to be formed first at the sides (fig. 35). I have no indication as to whether the free flagellum is absorbed (retracted) or breaks off; the encysting parasites of figs. 34, 36, 37 may have been, of course, forms without any free portion of the flagellum.

Fig. 34—37.



Fig. 38—41.



Fig. C. All figs. $\times 2250$. (By an error these have been reduced rather more than is intended.) Encystment. 34—37, different stages in the process; 38—41, complete cysts.

When I examined these infected female mosquitoes in the spring, not foreknowing of the occurrence of cysts, the subsequent natural destiny of the "Crithidia" appeared very debatable. I kept two living preparations of "rejuvenated", active Flagellates, from two digestive tracts, one lot in water, the other in normal salt-solution. After 24 hours, only a small number of active individuals were still apparent; others were very languid, the flagellum moving feebly; a large number had died or disintegrated. After 48 hours, only a few solitary individuals could be found alive in the aqueous preparation, free in the water. But the interesting fact was noticed that a considerable number of parasites which had remained enclosed inside a portion of the digestive tract, namely the pyloric end of the stomach and the proximal region of the intestine (which had been left in the preparation), were still quite normal and active at this period; and several of these were still alive the following day, when there was no sign of living individuals in the water around. These observations certainly appear to indicate that the active parasites are not able to live for any length of time in water, outside the host; and this is only what was to be expected, bearing in mind that they were not encysted. There is another fact which makes it difficult to suppose that these individuals succeed in being taken up by larvae. In the great majority of cases, at any rate, there can be no larvae available to

act as hosts, at the time when the parasites would be passed out to the outer world. For the hibernating females have first to develop and ripen their eggs, which have to be laid and undergo further development, before there can be any larvae about. One of the first things a hibernating female does, on waking up in the spring, is to have a meal of blood; for the eggs of *Culex pipiens* will not develop without blood, of this I have assured myself. Undoubtedly, after a female had partaken of blood, a large number of the parasites would be passed out to the exterior with the first faecal evacuations, for some faeces are evacuated very soon after a meal, especially if the mosquito has gorged herself. So far as can be seen, therefore, a great proportion of the parasites must perish. Before I knew of the occurrence of cysts, it seemed to me that it was not by the expulsion of the Flagellates that the survival of the species was ensured; and, as will be mentioned below, another mode of dispersal appeared possible. Now, however, that I have found that encystment does take place, it appears probable that the cysts are destined to infect larvae. Nevertheless, even if this is the case, the chances against successful larval infection seem to be very great. This is shown by the following fact. After the females had all disappeared from his cellar, Mr. Bacot placed receptacles containing water in his garden, for the larvae to develop in. He brought me up numbers of larvae, of different ages and certainly from different broods, at intervals from these receptacles. All the larvae, without exception, were of *Culex pipiens*. I examined a number and not in a single case have I seen any signs of a Flagellate, whether in the active or resting condition; and it may be reasonably supposed that some of these larvae, at any rate, were the offspring of one or more of the infected females. I may also repeat here, what I have noted on several previous occasions, that I must by now have examined altogether hundreds of larvae and newly emerged imagines, both male and female, of *C. pipiens*, from various sources within a short distance of each other in this same locality, during the last few years, without ever coming upon a Flagellate!

Having regard to the observations recorded above, there are one or two tentative possibilities bearing upon the subsequent history of these flagellate parasites which should not, I think, be overlooked. And this brings me to a subject which I wish to discuss shortly, namely the question of the connection or otherwise of "*Crithidia*" *fasciculata* with some Trypanosome. *Culex pipiens* is essentially the British mosquito which likes Avian blood; and propos of this point, I may mention that a week or so after Mr. Bacot brought me the last hibernating females, he captured an individual in his garden which contained fresh blood. This I examined and found to be Avian blood. Now one poss

lity is that faeces containing the parasites may be evacuated while the mosquito is in the act of feeding, and dropping on to some exposed part in the neighbourhood of the eye or nostril, where the mosquito usually feeds), thus bring about an infection of the bird. We know now of at least one instance where a Trypanosome-infection is brought about by means of the faeces. If this does happen in the present case also an explanation would be furnished of the apparent enormous waste of un-cysted individuals. On the other hand, it is quite likely that, as the resting Flagellates would doubtless become active again as soon as fresh fluid (blood) reached the intestine, some of the parasites would be able to pass forwards into the stomach and repopulate it. There is then, of course, the alternative hypothesis that certain of these Flagellates could in turn produce inoculative forms, which could infect a bird (if the right host) at a subsequent meal.

Up to the present, I have not been able to obtain experimental evidence to shew whether either of these possibilities actually occurs. It has been clearly proved, however, by Novy, McNeal and Torrey (1911) that "*Crithidia*" *fasciculata* thrives in the mosquitoes in the presence of blood. They state that the Flagellates occurred most abundantly in the stomach at from 40 to 60 hours after feeding, having multiplied rapidly during this interval and "permeated" the stomach. (The mosquitoes used were wild ones, and were fed on pigeon's or guinea-pig's blood.) On the other hand, Patton⁶ has pointed out that a true *Leptomonas* of mosquitoes (*C. fatigans*) is readily found in the male individuals, but only rarely in the females, the reason being that when the latter are fed on blood, the parasites usually disappear (after having been present in the larvae).

With regard to the origin of the Flagellates in the infected hibernating mosquitoes, there are one or two interesting bionomical points bearing upon the question which I may mention. I was able to make a few personal observations during the early summer upon the relation between the food of the female and the development of her eggs and their oviposition. It is possible, however, that a distinction must be made in this connection between "summer"-females, which produce larvae during the season, and "autumn" ones, which hibernate and produce larvae the following year. I found that the former individuals could always take a meal of blood (the conditions being suitable, of course) before being fertilized; indeed, none of the females which I have examined soon after a (first) meal of blood has been fertilized. This applies, however, to females bred in captivity and I am not at all certain

⁶ Sci. Mem. Med. India, No. 53. 1912.

whether the same applies to "wild" individuals. At any rate, the paired masses of eggs develop to their full size, ready for fertilization, upon one meal of blood. This summer, I have succeeded in obtaining fertile egg-rafts and larvae from bred-out males and females. I have a strong idea that a second meal of blood is taken normally before the fertile eggs are laid, the eggs being laid, in fact, immediately after the meal, which has perhaps induced the oviposition. I have found this to be the case in at least two instances; and, on the other hand, I have noticed that a gravid female, which has not fed again, will occasionally lay her eggs, but these have not been fertilized. A point to which attention must be drawn is that, as a result of a meal of blood, the eggs always do grow, apparently to their full size.

Now, in the hibernating female mosquitoes, the eggs apparent in the ovarian tubes are quite young (the females were, of course, fertilized). There are, it would seem, two alternative explanations. (a.) These individuals had never taken blood. If this were the case, it is obvious that the "*Crithidia*" could not have developed directly from a blood-Trypanosome. (b.) After one or more meals of blood, they had developed and laid a batch of eggs, and the young eggs present represented a succeeding batch. In none of the individuals I dissected could I obtain any definite indication which of the two interpretations was the correct one. But within the last few weeks, Major Perry, I. M. S., who in my absence was examining some females which had entered upon hibernation this (present) autumn, in the same cellar, found in one case a single, full-sized egg, in addition to the customary small ones; *i. e.*, one of the preceding batch which had been left behind when the rest were laid. This shews at any rate that the second alternative noted above does happen; in other words, that these hibernating females may have taken blood. And that is as far as I have been able to carry the problem up to the present.

From the above considerations, it still appears to me quite likely that "*Crithidia fasciculata*" is connected with a Trypanosome. Nevertheless, in view of the occurrence of cysts which are probably destined to infect the larvae, it is equally possible that this parasite is solely an Insectan Flagellate, that is, one restricted to the mosquito, which has become adapted to the sanguivorous habit of the female; I may recall that I expressly indicated the possible occurrence of such forms some years ago, though this suggestion of mine has been wilfully overlooked by some of my critics⁷.

Up to the present, there is no instance which is definitely established

⁷ Vide Lankester's treatise on Zoology, pt. 1, fasc. 1. Art: Haemoflagellates, p. 244.

the developmental forms of a Vertebrate Trypanosome producing cysts in the Insectan host, for expulsion to the exterior. But in the case of *Trypanosoma grayi*, in *Gl. palpalis*, it is not improbable, I think, that we have such an instance. It may be pointed out that among the figures given by Kleine and Taute⁸ of the forms of *T. grayi* in the tsetse-fly, which were proved by the authors to be derived from the trypanosome in the crocodile, there are two (figs. 65, 67) of the slender, so-called) herpetomonad type, which were shown by Minchin⁹ to give rise to the cysts. The German workers, it is true, did not observe any actual cyst-formation. Just as "*Crithidia*" *fasciculata* has been seen many times, however, when the cysts have not been found, so it has been with *T. grayi*; thus Roubaud¹⁰ only found cysts on 13 out of 30 occasions, in tsetses infected with *T. grayi*. The last-named author confidently assumes that this parasite is purely an Insectan form; but the series of forms which he figures agree entirely both with those given by Minchin and with those given by Kleine and Taute, — forms which in my opinion do not resemble the types of form generally found in the herpetomonads or trypanosomids of Insects.

There is an important hypothetical point which may be mentioned in this connection. The formation of cysts by a parasite occurring in a blood-sucking Insect does not necessarily mean that this parasite is unconnected with a Vertebrate Trypanosome. For in the case of a Trypanosome derived originally from an Insectan Flagellate, one which most probably formed cysts for its transmission from Insect to Insect (or larva), it is quite comprehensible that in certain cases this primitive mode of transmission may have been retained, in addition to the (secondary) method of transmission by inoculation to the Vertebrate (or possibly even without the latter), where this course proved advantageous to the species. If "*Crithidia*" *fasciculata* is really the developmental form of a Trypanosome, such an explanation would account for the presence of resistant cysts. Of course, this is nothing more than a hypothesis so far; but it is interesting to note that a similar view has been suggested by Nagas¹¹, in his account of *Trypanosoma* (*Schizotrypanum*) *cruzi*. He puts forward the possibility of the infection of fresh bugs (*Conorhinus*) by means of the excrement of infected ones, and is inclined to suppose this may be effected by the "crithidial" forms of the parasite (without the occurrence of actual cysts being suggested).

In conclusion, although the fact of the occurrence of cysts in

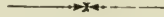
⁸ Arb. kais. Gesundheitsamt, 31. 1911. S. 321. 5 pls.

⁹ Quart. Journ. micr. Sci., 52. 1908. p. 159. 6 pls.

¹⁰ C. R. Soc. Biol. 72. 1912. p. 440.

¹¹ Mem. Inst. Oswaldo Cruz, 1. 1909. p. 159. pls.

"*Crithidia*" *fasciculata*, is one which, per se, argues in favour of the Insectan Flagellate view, having regard to the observations and suggestion brought forward above, I regard the question whether or not this parasite is connected with a Trypanosome as, at any rate, still an open one; and I consider the case of *T. grayi* is in a similar position.





Further remarks on the flagellate parasites of *Culex*. Is there a generic
type, *Crithidia*?

By H. M. Woodcock, D. Sc., Lister Institute of Preventive Medicine, London.

(With 1 figure.)

In a preceding Note in this Journal (Zool. Anz. Vol. 53, No. 8, 370). I have described the various forms of a parasite found in hibernating *Culex pipiens*, which I have regarded as "*Crithidia fasciculata* Léger (1). These forms agree on the whole closely with those described by Novy, McNeal and Torrey (2) from the same insect, which they also refer to Léger's parasite. I pointed out, however, that I was quite unable to see anything that could be safely interpreted as an undulating membrane, either from the movements of the parasites, both short and elongated forms, or from their appearance in fixed and stained preparations. In regard to this point, the account of the American authors is somewhat confusing. In one place they say that in this form the existence of such an organella could not be satisfactorily established. A little further on, however, they state that, in certain of the longer individuals, a distinct wave-motion at the anterior end could be seen in life, giving evidence of the presence of a membrane; and they conclude finally that this parasite ("*C. fasciculata*") has an imperfect (i. e. rudimentary) membrane. Having regard to these last definite statements of Novy, McNeal and Torrey, I considered that probably the reason why none of my forms shewed any membrane was because they occurred in fasting females and were only rejuvenated, as were, into activity by the addition of the fluid in which they were examined; whereas those investigated by the American authors were always examined at a period when some hours had elapsed after a meal of blood, when the parasites were swarming in the stomach. For there can be no doubt, I think, that both they and I have been dealing with the same form.

On the other hand, Léger, in his original account of *C. fasciculata*, figured a phase with a quite unmistakeable membrane, extending along part of the body and for some distance along the flagellum, as belonging to this parasite. The American workers suggested, as an explanation to account for this discrepancy, that Léger was really dealing with a mixed infection and had included phases belonging to distinct parasites in his description; this view was also taken by Lutton (3). As supporting their suggestion, Novy, McNeal and Torrey shewed clearly (so far as can be judged) that a mixed infection does occur in the "wild" *Culex*, and separated a parasite which they

term *Trypanosoma* (*Herpetomonas*) *culicis* from *Crithidia fasciculata*, both on morphological grounds and by means of cultivation. Adopting this view, it would result that Léger's *fasciculata*, the type-species¹, was a small form, with only a rudimentary membrane, and lacked the elongated phase with wavy membrane shewn by many other crithidial parasites.

On further consideration of the whole subject, however, and especially since, for the purpose of this note, I have been comparing the various phases of the different "Crithidia" which have been described, I prefer another explanation, which is, I think, much more probable. In the first place, I can see no reason for concluding that Léger has indeed described more than one form in his account of "*Crithidia*" *fasciculata*. This parasite is by no means the only form with an undulating membrane, i. e. a *Crithidia*, which possesses a short, oval or pear-shaped, so-called "gregariniiform" phase, serving for attachment (I propose to call this the haptomonad phase). Thus both the "*Crithidia*" *minuta* and "*C.*" *subulata* (which latter is not a *Herpetomonas*) subsequently described by Léger shew very similar stages in their life-history, certain individuals having just the same truncated appearance shewn by many of the small forms of *fasciculata*; and so has equally the "*C.*" sp. described by Patton (4) from *Tabanus* sp. In view of this I fail to understand why Patton should have thought it necessary to suppose that Léger's small forms of "*C.*" *fasciculata* should belong to a *Herpetomonad* (rather a *Leptomonad*, see below) and not to the *Crithidia*; particularly when, as he has himself specially pointed out, it cannot be determined from the short, haptomonad phase alone whether a parasite is a *Crithidia* or a *Leptomonad*, since in both this phase is essentially of the same type, with the two nuclei usually close together and the rhizoplastic part of the flagellum drawn back. Contrary to the opinion both of the American authors and of Patton, I think it most likely that all the forms described by Léger under the name *fasciculata* do belong to that parasite, because they form a regular and connected series. This being so, it appears also most probable, in the second place, that the parasite from *Culex pipiens* which the American workers and myself have had under observation is not Léger's actual form "*C.*" *fasciculata*, but is on the contrary preferably regarded, so far as can be judged from the phases at present known, as a *Leptomonad* rather than a *Crithidia*.

Before going further, however, a brief explanation is desirable as to why I use the term *Leptomonad*, and not *Herpetomonad*, in this connection. Hitherto, bot

¹ The specific name *fasciculata* would have to be applied, of course, to the parasite first described, i. e. the small "crithidial" form.

These terms have been used, often more or less indiscriminately, to denote a type which, in the elongated, monadine form, has typically the kinonucleus near the anterior end of the body and well separated from the trophonucleus, and concurrently, the flagellum springing directly from the anterior end of the body, becoming at once free; it follows from this that there is no trace of a membrane, the flagellum being connected with the body only by a short rhizoplastic portion. *Herpetomonas* *muscae-domesticae*, the type-species of this genus, was considered by Prowazek and others to possess a double flagellum (i. e. to be biflagellate); the species of *Leptomonas*, on the other hand, have only a single flagellum. Occurring associated with *H. m.-d.*

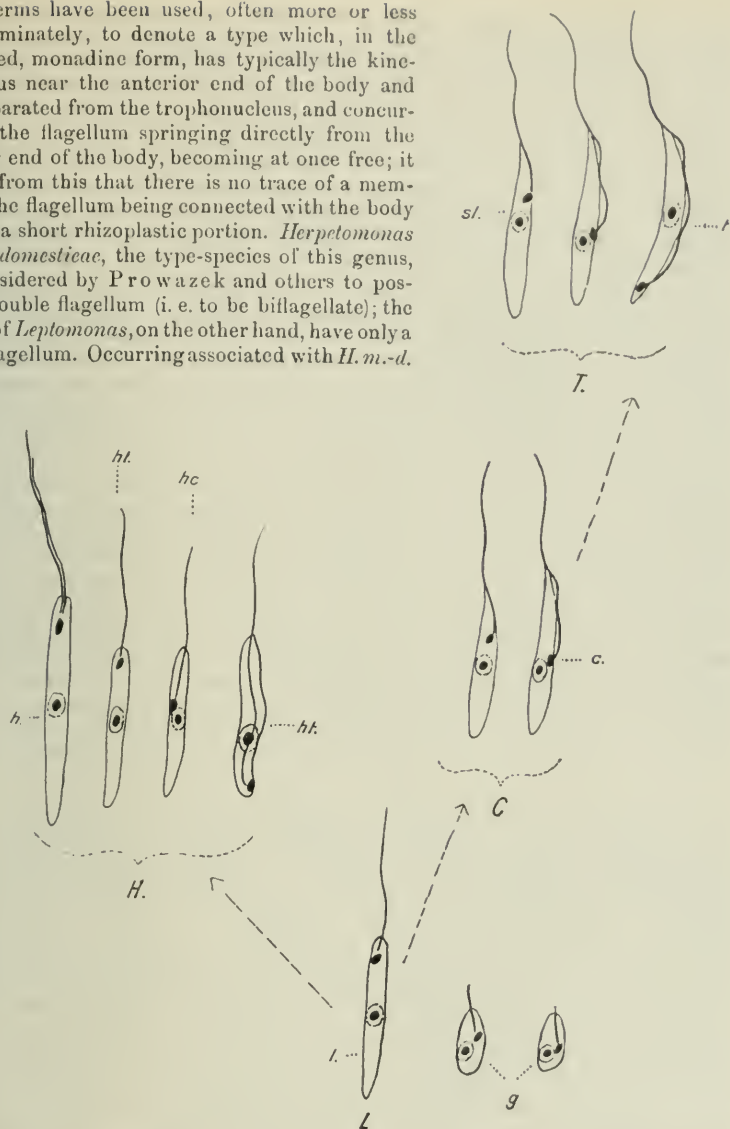


Fig. 1. Scheme illustrating the relationships of the different types discussed in the text. (For the sake of completeness it may be added that the figures of *Leptomonas* would serve also for *Leishmania*.)

— = *Leptomonas*; H = *Herpetomonas*; C = *Crithidia*; T = *Trypanosoma*. l , leptomonad form; g , haptomonad (so-called gregariniiform) phase for attachment, possessed by all four types; h , herpetomonad form; and hl , leptomonad form of the *herpetomonas*. (The distinction between these two phases is chiefly one of size and reciprocal division of the flagellum and is probably not so manifest in many cases.) c , crithidial (or "crithidiiform") phase of H ; hl , herpetotrypaniform phase ("trypanoid"); c , crithidial form (or "trypanomonad" in the case of *Trypanosoma*); sl , semi-leptomonad phase of *Crithidia* or *Trypanosoma*; t , trypaniform phase of T .

in house-flies, such a *Leptomonas* (a smaller form) has been described by various workers (e. g. Roubaud, Flu, Rosenbusch), which possesses in addition crithidial and trypanosome-like phases. This latter parasite is quite comparable in short to the various Leptomonads with leptotrypaniform phases (trypanoids), which have been described especially by Roubaud, Chatton and their co-workers from African flies (e. g. *Drosophila* sp.). Most of these authors have regarded the two forms as distinct parasites and have retained the generic name *Herpetomonas* for the large form and adopted that of *Leptomonas* for the other type. Dunkerly (5) has recently given a very good account of both forms from British house-flies, and while not able to connect them definitely, has suggested the possibility of their being both different forms of one parasite. Lastly, Wenyon (6) has just published a paper on this subject in which he shews clearly that all the different forms actually belong to the life-cycle of one parasite. He proposes to retain the generic name *Herpetomonas* for this parasite of house-flies, and to use that of *Leptomonas* for those Leptomonads (with a single flagellum, of course) which have not, so far as is known, any crithidial or trypanosome-like phase in their life-cycle. With this view I agree entirely. For, as he points out, although we do not yet know whether the type-species of this latter genus (*L. bütschlii*) possesses these additional phases, it is quite as likely that it lacks them, for several parasites are now known which certainly seem not to have them (e. g. the parasites described as *Herpetomonas jaculum*, *tygaci*, *aspongopi*, to name only a few). All these are best placed provisionally in the genus *Leptomonas*, as *L. jaculum*, and so on. On the other hand, all the forms of Roubaud, Chatton and others, which possess crithidiform phases and trypanoids (or "herpetotrypaniform" phases), come in the genus *Herpetomonas*. Because, in addition to connecting the large *Herpetomonas* of the house-fly with the smaller (*Leptomonas* form, Wenyon (l. c.) has come to the conclusion that the first-named is not to be regarded as really biflagellate, but as possessing a single flagellum which is frequently found precociously divided. Chatton himself, in one of his more recent papers (7) also expressed the same opinion and considered that *H. muscae-domesticae* and his *L. drosophilae* and other sp. were not so separate as had been formerly thought. Hence, it is best to write *Herpetomonas drosophilae*, *mcnili* and so on.

To return to the discussion of the parasite from *Culex pipiens*, I have referred above to the reasons which particularly influenced me in continuing to regard it as a "*Crithidia*". I think now that I did not attach sufficient weight perhaps, to the occurrence, in the infections which I studied, of certain forms which can hardly be regarded as other than Leptomonads (ante Herpetomonads) (cf., for example, my fig. 31). It is true that these individuals are very scanty in number, most of the elongated forms being, as I pointed out in my previous note, not typical Leptomonads, but differing in having the anterior end of the body more or less tapering, the flagellum being consequently attached to the body for a greater or less distance, this depending also, of course, on the exact position of the kinetonucleus (cf. my figs. 16, 17, 29, 32, 33 and also the American workers' fig. 4 pl. 8 of a rosette). Many of these forms resemble certain which develop in cultures of Avian Trypanosomes (e. g. *T. fringillinarum*), which I have distinguished in my first memoir on Avian Haemoprotozoa (8) as "pseudoherpetomonad" forms (I prefer to term them in future "semi-leptomonad" forms). It is just in such a case, of course, that it is difficult to decide whether to regard a parasite

is crithidial or leptomonad. It is entirely a question of degree; for such a condition is transitional between a typical *Crithidia* and a typical *Leptomonas*, and may be quite as readily connected with the one type as with the other — if anything, indeed, more readily with the former.

On the other hand, so far as I have been able to ascertain from a comparison of the different species described, no "*Crithidia*" shows a true leptomonad phase, that is to say, of course, in the elongated, monadine condition. I think this is a most important point, and one to be borne in mind when we try to distinguish a generic type, *Crithidia*. While one would not go so far as to say that all Crithidiæ entirely lack such a phase, (remembering that the crithidial type is derived from a leptomonad one), still, its absence appears to be a very general feature. As already indicated, I have no reason to suppose that the few Leptomonad individuals which I found represent a form distinct from the other phases; everything points to their belonging to the life-cycle of one and the same parasite. It seems best, therefore, to regard this parasite from *Culex pipiens* for the time being as a *Leptomonas*, its name becoming *L. fasciculata* (= *Crithidia* f. N. McN., and T., nec Léger). This implies, of course, that it does not really possess any undulating membrane². I find that Patton, in the two papers already referred to (3 and 4), has also expressed the same opinion with regard to the form studied by the American workers³.

Before leaving the subject of the flagellate parasites of *Culex*, a few observations may be noted with regard to certain other forms which have been described. As mentioned above, Novy, McNeal and Torrey gave at the same time an account of another parasite from *C. pipiens* and other sp. which they called *Trypanosoma* (*Herpetomonas*) *ulicis*, n. sp. As Patton has also pointed out, a typical *Crithidia* as now understood (with well-developed membrane) is concerned here; in this case the authors' description and figures leave no doubt upon the matter. This form is certainly not a *Herpetomonas* (*Leptomonas*) at all. Of course, in the phase described, it is not a true Trypanosome, because the kinetonucleus and the origin of the flagellum are not near the flagellar end of the body. But for all that, it is quite likely that this

² This certainly renders it less likely that this parasite is connected with a trypanosome; but does not, of course, affect the question of "*Crithidia*" *fasciculata*.

³ I may add, however, that I had come to the conclusion indicated quite independently, as a result of my own work, and before reading Patton's earlier remarks on this parasite. As will be apparent from what has been written above, one had not sufficient evidence, from a consideration of Novy, McNeal and Torrey's account alone, to regard this form as a Leptomonad rather than a *Crithidia*, any more than one has to say that the small forms of Léger's "*C.*" *fasciculata* do not belong to the same parasite as the larger (monadine) individuals.

parasite is really the Insectan phase of some Trypanosome⁴. Patton considers that this form is identical with Léger's "*C.*" *fasciculata*. I am rather inclined to regard it as a distinct parasite, whether one associates it with a Trypanosome or not; because Novy, McNeal and Torrey did not find in connection with it the characteristic "grain d'orge" phase shewn by Léger's form and by various other Crithidia. (This is assuming, of course, that the American workers were correct in separating their crithidial parasite from the small forms above discussed.) Hence I prefer to retain the name "*C.*" *culicis* (N., McN. and T.) for this parasite for the present.

Patton has recently given a detailed account (9) of a Leptomonad parasite from *C. fatigans* in India; this is a quite typical *Leptomonas* (or uniflagellate *Herpetomonas*, as hitherto understood). I must say that, in this paper, Patton appears to have done his best to thoroughly confuse the subject of "*Crithidia*" and *Leptomonas*, as occurring in mosquitoes. Patton actually refers his parasite to Novy, McNeal and Torrey's form, *Herpetomonas culicis*, although he himself has previously recognized that this latter form is a typical *Crithidia*! Until I had looked through his earlier papers I was quite at a loss to imagine whatever he meant. One can only suppose that Patton has calmly transferred the specific name of the *Crithidia*, viz. *culicis*, to the Leptomonad form of the American workers, in utter disregard of the established rules of nomenclature, according to which the parasite to which the name *culicis* has been given must retain that specific name, even though it be a *Crithidia*, and not a "*Herpetomonas*" as the American authors considered; just as, similarly, the Leptomonad form must still bear the specific name *fasciculata* bestowed upon it by the American writers. But there is no mention of this juggling with specific names in Patton's paper. Readers are left entirely under the impression that he is dealing with the parasite described as *Herpetomonas culicis* by Novy, McNeal and Torrey. In summarizing their observations he uses throughout the terms *H. culicis* and *Crithidia fasciculata* just as the Americans used them, saying, for instance, that they found so many mosquitoes to be infected with *Crithidia*, so many with *Herpetomonas*, and so on; (the latter generic name should certainly read *Crithidia*, and the former preferably *Leptomonas*). In the whole of his detailed account, I can find no mention whatever of the fact that he is not dealing actually with Novy, McNeal and Torrey's *Herpetomonas culicis* at all, which is a *Crithidia*, but with a quite different parasite. One can scarcely imagine

⁴ It must be remembered that all the *Culex* investigated by the American authors were "wild", i. e. caught individuals.

nothing more liable to mislead others upon the already sufficiently confused and difficult subject of the nomenclature of these forms.

I consider this parasite from *C. fatigans* is most probably a species distinct from *Leptomonas fasciculata* of *C. pipiens*. In the first place, the two hosts have a quite different distribution, and this is a factor which I have always maintained must be taken into account. Again, the elongated, monadine individuals of Patton's parasite are considerably larger than any of *fasciculata* which I have found, or which are described and figured by the American authors. Moreover, although both parasites appear to be of the same general type, the monadine forms of the parasite from *C. fatigans* are more typically leptomonad than are, for the most part, those of *fasciculata*, as I have discussed above. On these grounds, therefore, the two are best regarded as separate species, and Patton's form should bear the name *L. culicis* n. sp. Patton (nec Novy, McNeal and Torrey).

It remains to add a few remarks upon the question of *Crithidia* as a generic type. We have, on the one hand, crithidiform (as well as herpetotrypaniform) phases occurring very generally in the life-cycle of *Leptomonas*; on the other hand, crithidial (or, as they are conveniently termed, trypanomonad) forms occur as a developmental phase in the life-cycle of most — perhaps all — Trypanosomes. Is there, therefore, a separate and independent generic type, *Crithidia*, which can be distinguished and characterized? While it is evident from the above facts that there is much to be said in favour of the view that crithidial forms represent only a phase in a life-cycle of one or other of the above types, I think, nevertheless, that there is sufficient evidence to make it at any rate very convenient to continue to recognize a distinct type, *Crithidia*. Leaving aside the many instances of crithidial forms occurring in blood-sucking Insects and the question of the connection of such parasites with some Trypanosome — a question which, I may point out, still remains in statu quo — there are a few forms which, it seems to me, may be regarded as furnishing the nucleus of such a genus. We have, for example, *C. campanulata*, *C. eleti* and *C. gerridis*. All these forms are parasitic in non-bloodsucking hosts. They possess the typical crithidial characters (undulating membrane, proximity of the two nuclei, etc.); and in neither is anything like a leptomonad phase (i. e. of course, the elongated, monadine forms) described. This last point seems to me to differentiate such a parasite from the crithidiform phase of a *Leptomonas*. So far as I gather from the accounts of various species of *H.*, when the crithidial forms are found, there is no difficulty in finding not only herpetotrypaniform individuals, but also the ordinary leptomonad forms; and Miss Robertson, for instance, in commenting

upon an infection of certain African bugs with a *Herpetomonas*, says (10) expressly that while the herpetomonad (or leptomonad) forms of the parasite were met with unaccompanied by crithidial forms, the converse was never observed. I think it is quite likely that many *Crithidia* may shew a semi-leptomonad phase, just as the crithidial forms of a *Trypanosome* may pass into such, either in cultures, or in the Invertebrate host; but that either the one or the other has a true leptomonad phase, comparable to that of a *Leptomonas*, seems to me to be doubtful; at any rate such a phase remains to be described. Provisionally, therefore, a *Crithidia* may be characterized as a form which possesses the typical crithidial features, enumerated above, which has not developed a trypaniform phase and which in most cases no longer possesses a typical leptomonad phase.

The relationships of the different generic types above discussed to one another are best indicated, it appears to me, not by representing the different forms in one phylogenetic line or series (thus, *Leptomonas* → *Herpetomonas* → *Crithidia* → *Trypanosoma*, or *Leptomonas* → *Crithidia* → *Herpetomonas* → *Trypanosoma*), but rather as comprising two distinct branches from a Leptomonad stock. For one can hardly suppose *Crithidia* to be derived from *Herpetomonas* by the loss of the herpetotrypaniform phase, only to give rise to *Trypanosoma* by the re-development of a similar phase again; and on the other hand, it is not likely that *Herpetomonas* with its well-marked, persistent leptomonad phase, has been developed through *Crithidia*. *Herpetomonas* most probably represents one branch or line of development from *Leptomonas*, *Crithidia* and *Trypanosoma* together, another. The idea may be expressed diagrammatically as in the accompanying text-figure 1.

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- 1) C. R. soc. biol. 54. 1902. p. 354. text-figs.
- 2) J. inf. diseases. 4. 1907. p. 223. 7 pls.
- 3) Arch. Protistenk. 12. 1908. p. 131. pl. 9.
- 4) Arch. Protistenk. 15. 1909. p. 333. pl. 30.
- 5) Q. J. micr. sci. 56. 1911. p. 645. pl. 31.
- 6) Arch. Protistenk. 31. 1913. p. 1. 3 pls.
- 7) C. R. soc. biol. 71. 1911. p. 578.
- 8) Q. J. micr. sci. 55. 1910. p. 641. 5 pls.
- 9) Sci. Mem. med. Ind. No. 57. 1912. 21 pp. 1 pl.
- 10) Proc. Roy. Soc. 85. 1912. p. 234. text-figs.



On a Remarkable New Type of Protistan Parasite.

By

H. M. Woodcock, D.Sc., and G. Lapage, B.Sc.

With Plates 29 and 30 and 2 Text-figures.

IN the course of a study of the flagellates, which develop in simple cultures of goat's dung, we were afforded recently the opportunity, by the kindness of Drs. E. H. Ross and J. W. Cropper, of examining the contents of the rumen and other parts of the digestive tract of a goat, with a view to ascertaining what flagellates occurred in the active condition in the goat. The results of our observations in this connection will be dealt with in another memoir. The object of this communication is to describe certain remarkable parasites which we found, which appear to be of a nature quite distinct from any other protist of which we are aware. These parasites occur under two characteristic forms, which we have distinguished respectively as crescents and ovals. We are strongly inclined to regard these two principal types as being different phases of one parasite. We have examined, up to the present, six goats, and in the rumen of each, either the crescents, or the ovals, or both forms have occurred in enormous numbers—far exceeding those of the ciliates or ordinary flagellates present. A comparison of the occurrence of the two forms is given in the following table:

Goat.	Crescents.	Ovals.
No. 1	Relatively infrequent	Abundant.
No. 2	Very abundant	Very abundant.
No. 3	Very abundant	Doubtful if present.
No. 4 (first time of examination)	Very scanty	Very abundant.
No. 4 (second time).	Much more frequent	Much less numerous.
No. 4 (third time) .	Numerous	Abundant.
No. 5 (first time) .	Present (see Note)	Present (see Note).
No. 5 (second time).	Fairly numerous	Numerous.
No. 6	Abundant	Very abundant.

Note.—The first three goats and the last one were killed, but in the case of the remaining two some of the fluid contents of the rumen were obtained by means of a stomach-tube. In the first examination of No. 5, only an extremely small quantity of material was obtained—scarcely any in fact—and this observation afforded no precise indication of the numbers of the parasite present.

Our attention was first directed particularly to the crescents because of their vigorous movements, and the fact that, in individuals which were moving but little or else were at rest, a single, conspicuous flagellum could be seen to be attached to the concave side of the parasite.¹ On account of this characteristic appearance we have given to this form the new generic name, *Selenomastix*. Before describing the parasite, however, we should point out that the crescents, at all events, have been undoubtedly observed before, for in the existing literature we are aware of two references which relate to this organism; but neither of them furnishes any true indication of its peculiar characters. The first record occurs in a short note by Certes ('Bull. Soc. Zool. France,' vol. xiv, 1889, p. 70), on the micro-organisms in the rumen of ruminants. This author observed, associated with the ciliates, a flagellate in the form of a crescent, which assumed at times an S-shape, and had its flagellum inserted at the middle

¹ We may add that the flagellum was actually observed first by Dr. E. H. Ross, in a preparation which he stained rapidly by the jelly-method, on being made acquainted with our discovery of the parasite.

of the incurved part of the body. The size is given as $8-9\ \mu$ long by $2-3\ \mu$ wide. Certes proposed the name *Ancyromonas ruminantium* for the parasite, although he recognised that there was very considerable difference between the new organism and the known species of *Ancyromonas*. (As will be seen from the subsequent account, the new parasite has nothing whatever to do with *Ancyromonas*.) Certes goes on to say that he found also in the rumen *Sarcinæ*, the predominating forms being ovoid, hyaline and small, about $8-10\ \mu$ by $2-3\ \mu$. The smallest forms showed sometimes the commencement of budding, and in consequence might be associated with yeasts. Others, the great majority in fact, multiplied by fission. The ovals which we have found have certainly nothing to do either with yeasts or *Sarcinæ*. It seems very probable, however, that they are the same thing as the predominating ovoids of Certes, which he erroneously connected with the smaller, budding organisms; these latter may have been of the nature of *Sarcinæ*. The second reference is a brief note by Kerandel, in a paper on hæmatozoa observed in the Congo ('Bull. Soc. Path. exot.,' vol. ii, 1909, p. 208), to the effect that he had observed from the œsophagus of an antelope (*Cephalophus* sp.) bodies similar to those previously noted by Certes. He refers to the conspicuous cilia inserted in the middle of the concave side of the body (cf. below, p. 440). Neither author gave any figures of the parasites, but it is apparent that both were dealing with the same creature which is here described. Hence, the new parasite must bear the name *Selenomastix ruminantium* (Certes).

The rumen is undoubtedly the principal habitat of *Selenomastix*. A small drop of the fluid contents taken from any part of this bulky organ (in the case of killed goats) has always been full of one or other form of the parasite. The crescents have been found also, in sparing numbers, in the rumen of a sheep. Hence this creature appears to be a common parasite of ruminants, and, at any rate, in the goat is very abundant. The parasites also occur in small numbers

in the reticulum, and a few have been seen in the true stomach, but they have never been observed in the intestine, cæcum or rectum. In the last-named region of the alimentary canal, cysts and spores of one kind and another occur, the determination of which is a very puzzling matter, and it is quite possible that some one of these represents a resistant phase of the parasites; but we have not succeeded in recognising anything corresponding to the characteristic ovals. Certainly no crescents are to be found, nor have they developed in any of the many cultures which we have made from the fæces, both of goats which we know to have been infected and of others which in all likelihood also were. In this feature *Selenomastix* agrees with the Ciliates and the ordinary Flagellates which occur in an active phase in the rumen. Like these, further, this new parasite appears to be purely an inhabitant of the fluid contents of the rumen. We have carefully examined the wall, both externally and internally, for any indication of cysts, large or small, but none have been visible. (None of the goats examined have shown any signs of the "cysts of Gilruth.")

We will first describe the appearance, behaviour and structure of the crescents, and can then readily compare the corresponding features in the ovals. Seen living and freshly removed from the rumen, either with or without the addition of a small drop of water or normal saline, all the crescents have a very similar and characteristic appearance, but show a considerable range of variation in size. The body has a uniform, homogeneous, dull-looking appearance. We have never observed the crescents alter in shape at all; they are certainly not amœboid or "metabolic," and we have never seen them assume an S-shaped form, as mentioned by Certes (cf. below, p. 439). Many, if not most, of the crescents possess a definite envelope, as is seen from stained preparations, but it does not stand out at all in life (contrast the ovals, below). No granules of any kind, or vacuoles, are noticeable in the protoplasm.

If examined as soon as possible after removal from the

rumen, a large proportion of the crescents are usually actively motile. As already mentioned, *Selenomastix* possesses a single,¹ relatively large flagellum, which appears to be inserted in the body, as a rule, about the middle of the concave side (Text-figs. A-E). The movements are very varied. We have distinguished the following kinds, but

TEXT-FIGS. A—G.



Crescents drawn either living or after fixation with osmic acid vapour. A-C seen from the side: D and E showing the origin of flagellum from concave face; F and G two different stages in division.

though we have studied them very closely we are in some cases not quite certain how they are caused. (1) The flagellum is directed behind and undoubtedly acts as a pulsellum, causing forward progression of the body, in a rather irregular, zig-zag, but not spiral manner. We have not observed a definite reversal of the direction of this movement, i. e. in the opposite sense, the other end of the creature suddenly leading the way. This appears to be the

¹ Many of the crescents have two flagella, but these are individuals undergoing division (Text-figs. F and G).

least common type of movement, but we regard it as very important, since it provides a distinct indication of antero-posterior polarity. (2) The body oscillates to and fro, or turns round completely, one and a half times, or even twice, in the plane of its long axis, i. e. turning a somersault as it were; no forward progression is effected. This movement may be quite rapid. When it can be seen, the flagellum stands out well from the body; it may cause this movement, but we feel undecided, for we have noticed that when a crescent is becoming a little sluggish and the oscillations take place at longer intervals and not so rapidly, a premonitory tremble of the body occurs the instant before the movement. (3) Some parasites, which are moving only at intervals, can be seen to turn slowly from one side to another, this time around the long axis. In these cases the flagellum appears to be quite passive, and to turn over after the body. We certainly consider this movement is caused by the body and not by the flagellum. Moreover, now and again a crescent can be seen oscillating more or less, when it is impossible to see a flagellum in connection with it, and though of course it is sometimes difficult to make out the flagellum, nevertheless, with the aid of good lenses and critical illumination, this can usually be clearly seen; further, crescents without a flagellum do undoubtedly occur. Hence we feel persuaded that movements caused by the body alone and not by the flagellum do take place (cf. also the ovals).

The movements of most of the crescents cease very soon after being removed from the rumen—surprisingly soon in some cases. After about an hour few are still to be found active, and their movements have become irregular and spasmodic. Even in a quantity of the fluid contents kept in a warm water-bath, at from 30° – 35° C., in three or four hours the parasites were all motionless. Crescents may have short resting intervals and may then become actively motile again; but when all or nearly all the crescents in a particular area of the drop under examination remain motionless, they may be regarded, we think, as being dead. In the rumen-contents

just referred to, though they were frequently examined during two days, we never found any active parasites after about four hours. The third goat examined furnished a remarkable illustration of this point. It was killed, and quantities of the rumen contents, taken from different parts, were put into two small dishes, which had been previously warmed. Not more than a quarter of an hour elapsed before these were examined, but although the crescents were extremely abundant, not one of them was active, though the flagellum could be made out in many. A warmed pipette was then taken and a fresh quantity of the contents obtained and immediately looked at; in the cover-slip preparations made here and there were areas of active parasites, though in other places they were motionless.

We consider that, in general, it is the lowering of the temperature which renders the parasites motionless, though we have come across exceptions. Thus, on one occasion, in a cover-slip preparation which had been kept at about 30° C. for twenty-four hours, a few parasites were still feebly motile. Again, in an endeavour to cultivate the parasites on agar plates, we have found two or three individuals still motile five days after being removed from the goat. In this character of extreme susceptibility to change of environment, *Selenomastix* agrees with the peculiar Ciliates and the Flagellates also present in the rumen; the Heterotrichous forms (*Eutodinium*, *Ophryoscolex*) are even more quickly rendered motionless—indeed, frequently one can no longer find an individual still active—while the Flagellates are apparently just about as susceptible as *Selenomastix*, remaining active for two or three hours. On the other hand, the motile bacteria which occur (bacilli, spirillar forms) remain active for a much longer time.

We may add here that no development of the parasites occurs when "cultivated" outside the body, so far as we have been able to ascertain. We have tried simple agar plates, varying the strength and consistency of the medium. There is no further multiplication or apparent increase in number

either of the crescents or of the ovals. Ovals can be recognised longer than the crescents and are probably more resistant (cf. below); but by the end of eight days the medium is so overrun by bacterial and fungal growths that nothing else can be made out. Dr. Ledingham, of the bacteriological department here, kindly had cultures of the parasites made for us on different media, and he also informed us that no development took place.

Structure.—*Selenomastix ruminantium* presents some highly remarkable features in its morphology and minute structure. The usual and typical form is slightly crescentic (figs. 1, 5, 7, 18); in the larger individuals it is often very like a banana (figs. 25, 26). The parasite is never sickle or S-shaped; and this is true however big and long the crescents may be. Further, in the vast majority of parasites, if not in all, the curve of the crescent lies in one plane, *i. e.* it is not spiral like that of a spirillum. Viewed in this plane the parasites have the appearance of considerably elongated ovals (figs. 8, 9). Nevertheless, now and again, but very rarely, one gets the impression of the slightest possible twist in the axis of the parasite; thus in fig. 21 and to a less extent in Text-fig. E, there is an indication of one end of the body pointing rather in the opposite direction to the other. We have looked particularly for indications of a spiral character of the organism in life, and this appearance, which we have observed in only very few individuals, is the only one we have obtained. Moreover, we do not feel at all certain that this appearance corresponds to a permanent twist, however slight, in the body. For we have noticed, in watching certain individuals progressing forwards—sometimes, too, individuals in which no flagellum could be made out—that the hinder part of the body moves slightly to and fro, laterally, in a zig-zag manner, distinctly more so than does the front part, which is kept fairly steady; this may perhaps be caused by a slight voluntary twisting of the hinder end of the body, first in one sense and then in the opposite one, this movement serving to propel the body (cf.

Certes' remarks, above, though there is never anything approaching an S-shape).

The concavity of the crescent may be only very slight (figs. 1, 14, 16), or may be practically absent (figs. 2, 4, 11, 12); small forms often appear thus. Individuals immediately resulting from division may be pyriform, differing from the ovals in having one end broader than the other (figs. 34, 35). We have not succeeded in finding an individual with both sides markedly convex, which at the same time possesses a flagellum; in other words, we have not found a typical oval with a flagellum. The nearest approach to an oval shape is seen in figs. 4 and 11, and these individuals, though they still come in the category of crescents (for one side is practically straight), nevertheless closely resemble certain ovals. When seen more or less in the plane of the curve, the body of a crescent can be distinguished from that of an oval by the fact that its ends are narrower and more tapering (figs. 8, 9).

The flagellum is apparently always attached to the concave (or straight) side of the body, and in the majority of cases its point of insertion is about the middle of this side. But this point varies to a certain extent, particularly in the smaller forms, where the flagellum may arise much nearer to one end (figs. 1, 3, also 18); we have never found it, however, actually terminal in origin. It is possible that this variation in the point of attachment of the flagellum may be partly dependent upon the process of division. The question of the orientation of the body is one of much difficulty. If the middle point of insertion of the flagellum represents approximately the anterior end, then it is obvious that the body is greatly extended laterally. It is certain, however, that the parasite never progresses forwards in a direction at right angles to its long axis, i. e. broadside on, as it were. We have, in fact, no reason to suppose that this is the right view to take. The only clue to an orientation of the body is the indication we get from certain movements of progression of the parasites of an antero-posterior polarity; in such cases

the end nearest to which the flagellum is inserted goes first and may be regarded as anterior, the flagellum itself being directed backwards.

As regards the dimensions of the parasites, crescents of average medium size have a length of 9.5 to 11 μ and a breadth of 2 to 3 μ (Text-figs. A-D and figs. 1-8, 11-16), the length of the flagellum being about 8-9.5 μ . The largest (single) individual we have observed (on a "wet-fixed" film) is 12.5 μ long by 3.25 μ broad and the flagellum is 15 μ long (fig. 10); on a "dry," Giemsa smear, the largest crescent found measures 13.5 μ in length by 3.75 μ in width,¹ the flagellum being 12 μ long (fig. 77). The smallest crescent observed, just in the act of separating after division (fig. 33), is only 4.25 μ by 1.9 μ ; another small one (fig. 35) is 6.25 μ long and rather stouter, being 2.5 μ broad. Between these extremes all intermediate sizes occur.

The flagellum itself may be as long as 16 μ (fig. 2) or as short as 7.5 μ (fig. 12); its length does not bear any very close relation to the size of the parasite, the small individual of fig. 5 having a very long flagellum, while the large parasite of fig. 19 has a relatively short one. A remarkable fact bearing upon the structure of the flagellum is brought out by "dry" Giemsa-stained smears. In wet-fixed films the flagellum does not apparently differ much from that of an ordinary flagellate; it has, perhaps, a thicker and stronger appearance on the whole, though it usually thins out a little and becomes more tapering towards the free end. On Giemsa smears, however, the flagellum is frequently seen to be more or less broken up into separate bands or fibrils, often throughout the greater part of its length (figs. 75-77); or else it has split into two or three fibrils near the free end. This appearance has certainly nothing to do with division, which is quite different (cf. below); moreover, we have never seen it in

¹ Some of the parasites on Giemsa smears are possibly a little too wide relatively, having been flattened out slightly in making the preparation; on the other hand, the parasites on wet-fixed films are probably slightly (uniformly) contracted.

wet-fixed films. Apparently the somewhat rougher treatment of the parasites in making a Giemsa smear—perhaps the drying—may cause the flagellum, in certain cases, to be partially broken up into component fibrils. This observation is very interesting, because it points to the flagellum having a structure rather different from that of most ordinary flagellates, for, in the course of our work on the forms which crop up in the faecal cultures (e. g. *Monas*, *Cercomonas*, *Bodo*), we have never observed such a splitting of the flagellum, and we have made numerous Giemsa smears.¹

It is apparent from wet-fixed preparations that a definite membrane or envelope surrounds many, if not all, the crescents. It is curious that, within a short distance of one another, parasites can be found, both single individuals and forms undergoing division, which show indications of this envelope to a very varying extent. Thus it may be seen, standing off from the general protoplasm of the body, only at one end (figs. 23, 35, 36); or at both ends (figs. 6, 16); or along one side (fig. 2); or nearly all round the body (figs. 3, 19, 27). In others, again, it is not discernible at all (cf. figs. 1, 4, 7). We are uncertain whether these different appearances represent the actual condition in life, or whether they are to some extent due to the body-protoplasm having undergone a certain amount of shrinkage away from the envelope in the wet-fixation, more especially in the direction of length. As mentioned above, no envelope, distinct from the general body-substance, can be made out in the living crescents, nor is it obvious, as a rule, in the parasites on Giemsa smears. A point to notice is that the envelope never stands off from the general protoplasm at the point where the flagellum is attached; this indicates that the latter organella is not merely a development from the membrane, but originates

¹ This splitting is never seen in the living parasites, but it is interesting to note that we have observed a somewhat similar splitting of the flagellum (in life) in a "true" flagellate occurring in the rumen (perhaps a *Sphaeromonas*), which possesses a long, thick, curved flagellum.

from the general protoplasm. We may say here that there is not the faintest hint of any groove or depression around the body of a crescent at the point where the flagellum starts.

Not the least remarkable feature of *Selenomastix* is its cytology. The best stain is undoubtedly iron-hæmatoxylin. Delafield's hæmatoxylin shows just the same minute structure, but it has the drawback that the flagellum is usually very faintly stained and often cannot be made out, and the same remark applies to carmine stains. Giemsa's stain is of considerable use in many respects, but not of much service in bringing out the details of the internal structure of the body, except where the chromatin is in the form of one or two prominent masses. The general protoplasm nearly always stains uniformly and homogeneously, sometimes lighter and sometimes darker, according to the degree of extraction. It never shows either granules of any kind or vacuoles.

There is no properly constituted nucleus, either of the usual karyosomatic type seen in the flagellates, or of any other type with which we are acquainted. Nevertheless, chromatin is undoubtedly present in greater or less quantity, occupying a peculiar but characteristic position. The principal situation of the chromatinic substance is at the periphery of the body. In the condition in which the parasite has apparently the least amount of chromatin, this constitutes a very narrow layer or zone, extending all over the surface of the body and appearing in optical section as a definite border, staining blacker and more intensely than the cytoplasm (figs. 3, 5, 6). More generally, however, this layer shows distinct thickenings, which may take the form either of numerous fine, small granules, appearing as little more than dots (figs. 1, 4, 8); or of few or several larger more conspicuous grains or small masses (figs. 9, 11, 10, 17); or, finally, of a few (usually one or two) quite large, dark-staining masses (figs. 12-16, 26). The granules and masses project inwards, one edge always being at the surface of the body, and we are inclined to consider them as having developed from the basal, peripheral zone or layer. Now and again these dark masses

form thick half-hoops or rings, partially encircling the body (figs. 18, 19). In large individuals they may occur together with conspicuous granules in the peripheral zone (fig. 25), but as a rule in the smaller forms, when a prominent chromatinic mass is present, the peripheral zone appears to contain very little chromatin.

Division.—Whenever the crescents have occurred in numbers we have found division proceeding actively. Division of the parasites always takes place by means of equal binary fission. We have never seen the slightest indication of unequal fission, or of anything in the nature of budding. So far as we have been able to ascertain, binary fission appears to be the only form of multiplication in *Selenomastix*. The division always takes place in a plane at right angles to the long axis of the body, i. e. it is transverse to it. Division does not stand, apparently, in any definite relation to the size of the parasite; that is to say, not only large individuals divide, but intermediate-sized ones and also quite small forms. Neither does the condition in which the chromatinic substance is present appear to determine fission, for individuals can be found undergoing division in which the chromatin is practically in any of the states described above (cf. figs. given of dividing forms).

In the great majority of cases, though not by any means always, the fission is initiated by the splitting of the flagellum along the greater part of its length. This is shown clearly in figs. 20, 23. There is no question of this appearance being merely a fraying-out of the flagellum into fibrils, such as was referred to above. For one thing, the instances figured (and others observed) are on wet-fixed films, in which the fraying-out is never found. Again, when the flagellum shows a frayed-out appearance, it is either the middle portion or else the free distal end which is split into fibrils of varying thickness; in the true splitting of the flagellum, leading to division, the basal part divides first of all into two daughter-flagella of equal thickness, the proximal, attached ends first separating. In the figures mentioned, the splitting has not

yet proceeded along the entire length, and the two daughter-flagella are still united into one distally. Probably the parent-flagellum splits nearly throughout its length, for the two daughter-flagella are usually approximately equal (figs. 24-28); it is exceptional to find a dividing individual in which there is as great a difference between the length of the separated flagella as in that of fig. 31. We have been considerably exercised in regard to the question whether there is a basal granule in connection with the flagellum. We are rather inclined to think that there may be such a granule, but we cannot say with certainty. There is frequently a definite granule exactly at the point where the flagellum originates (figs. 11, 13, 21 and 22), but owing to the peripheral situation of the chromatinic zone, it is possible, of course, that such granule is a chromatin grain. Nevertheless, in such a case as is shown in fig. 20, where the chromatinic zone is very feebly developed, but where there is a very distinct granule-like thickening at the basal end of each of the daughter-flagella, definite basal granules are certainly suggested.

As regards the division of the chromatinic substance, we have found nothing to indicate that there is any pronounced attempt at equal distribution between the two daughter-individuals. Apparently, the chromatic substance which happens to be in either half, prior to division, goes to that daughter-individual (cf. figs. 24 and 25). However, the most usual condition in which the chromatin occurs in dividing individuals is that of a number of small granules fairly uniformly distributed around the periphery (figs. 27, 28), and therefore there may be really a nearer approach to equalisation than is obvious owing to the absence of a definite nucleus. The last act in the process is the constriction of the general body-substance into two halves; this always takes place exactly in the middle of the long axis.

In some cases fission of the body undoubtedly occurs before the flagellum has split (figs. 32, 30, 29 show different stages in such a process). Whether the daughter-individual which thus lacks a flagellum is able to develop one we cannot say,

but crescents which do not possess a flagellum certainly occur (figs. 36, 37). We are inclined to think that such forms may become ovals, which we have next to consider.

The Ovals.—In their general appearance the ovals resemble the crescents. Their average size also is quite comparable; they are somewhat shorter, but distinctly more bulky. Some of them are seen to be considerably elongated, but these are individuals either about to divide or in the act of dividing. Apart from their shape the essential point of difference from the crescents is that the ovals entirely lack the characteristic flagellum. Nevertheless the ovals are undoubtedly capable of movement, and this fact was brought home to us in a surprising manner. When the fourth goat was examined for the first time an enormous number of active ovals were found, while the crescents were extremely scanty—far fewer in number than on any other occasion. The great majority of the ovals were in motion, the movement being one of progression, in a slightly zig-zag manner, but no sudden reversal of the direction of movement was noticed. At each of the subsequent examinations of the same goat, when the ovals have been relatively fewer and the crescents more numerous, most of the ovals have been quite still (although there were active crescents in the same preparations). Here and there, however, an oval would be seen zig-zagging to and fro slightly and spasmodically, scarcely progressing at all. And this has been the case in most of the other goats examined; nearly all the ovals were motionless. We have not observed any rotation of the ovals on their own axis, such as is commonly seen in the crescents. The remarkable activity of the ovals on the particular occasion referred to soon subsided, and after about a couple of hours they were all still. In the last goat examined, however, many of the ovals, as well as the crescents, were active, and we were able to observe a distinct indication of antero-posterior polarity in their case also. Now and again an oval steadily progressing would come against an obstacle. When this occurred the oval did not move away in the opposite sense, but turned

quite round and went off in another direction, the same end still being in front.

We had some of these active ovals specially stained for us by one of the principal methods in use (de Rossi's) for showing up the flagella of bacteria, but with no result whatever. We have ourselves tried this method and also Pitfield's method, with equally negative results. In short, in none of our preparations, however stained, have we ever seen a flagellum or tuft of flagella in connection with an oval; and we feel convinced that the ovals do not possess flagella of any kind. We are supported in this view by two points: (1) The close agreement in minute structure shown by many of the ovals and crescents, and the fact that the flagellum of the latter is a well-developed structure, readily visible; and (2) the conviction we have gained that the crescents themselves are capable of movement by other means than their flagellum.¹

While many of the ovals have the same homogeneous appearance in life as the crescents, in some the membrane or envelope stands out distinctly, being separated from the general body-substance by a narrow, clear area (Text-figs. H, L and M). In the majority of the ovals, the envelope appears to be more prominent and more distinct from the body than in the crescents, and this is borne out by the study of stained preparations. Probably it is a firmer, more resistant structure in the ovals.

Structure.—The ovals are rarely, if ever, spherical; the nearest approach to a spherical shape is seen in forms immediately resulting from division (figs. 44, 55, 57), and even in these one diameter is usually greater than the other. On the other hand, they are rarely sufficiently long in proportion to their width, and the two longer sides sufficiently straight and parallel, for them to be regarded as rod-like; here, again, the nearest approach to such an appearance is shown by those forms about to divide (fig. 56). Undoubtedly the oval

¹ It may be added, perhaps, that neither do the crescents show any flagella of the bacterial type when stained by the above-mentioned special methods.

shape is typical of this phase of the parasite. The average size varies from $7-9\frac{1}{2}\mu$ in length, by $3\frac{1}{2}-5\mu$ in width; individuals with dimensions less than these occur, but larger ones are nearly always in the act of dividing; the individual of fig. 48 is about the largest single oval found.

In the case of the ovals, the minute structure can be made out satisfactorily only in wet-fixed preparations, stained by hæmatoxylin; in Giemsa smears, the ovals—especially the large ones—stain much more intensely than the crescents, and usually appear rather blotchy, the stain being deposited to a greater extent either in, or immediately beneath, the (thicker) envelope. Hence, only two or three examples

TEXT-FIGS. H-M.



Ovals drawn either living or after fixation with osmic acid vapour.

H, L and M show the envelope distinctly; in J and K it is not visible.

stained in this manner are figured, for the sake of comparison (figs. 83-87). The ovals show two types of minute structure, which, at first sight, appear quite different; we think, however, that they are connected by intermediate conditions. The first type of structure is practically identical with that of many crescents. There is just the same difference with regard to the distinctness of the envelope. In many individuals it stands off well from the general protoplasm at the two ends of the body (figs. 39, 40, 53); in others, though these are fewer in number, it is not apparent at all (figs. 41, 43, 45, 54). The protoplasm stains in the same uniform manner and shows neither granules nor vacuoles. The chromatinic substance is distributed in the same characteristic manner, constituting usually a zone of

fine granules closely arranged round the periphery (figs. 39-41), or, more rarely, comprising fewer, somewhat more prominent granules (figs. 45, 46, 49). We have not found any ovals with one or two large, deeply staining masses of chromatin such as are shown by some of the crescents. Further, the ovals divide in just the same way, by equal, transverse, binary fission (figs. 49-53). Whatever the crescents are, we think there can be no doubt that these ovals are, at any rate, a very similar type of thing (cf. especially figs. 6, 27 of crescents with figs. 40, 53 of ovals); the only essential point of difference is that the latter have no flagellum.

In the great majority of the ovals which show the second type of minute structure, the envelope projects markedly at both ends of the body (figs. 65-69), and now and again it stands off slightly also at the sides (figs. 64, 66, and fig. 87 on a Giemsa smear). The general protoplasm is usually sharply divided into two distinct zones, a central, lighter-staining region and a peripheral, more deeply staining area, which is usually wider at the two ends. The lighter staining, central area appears very similar to the general cytoplasm of the other ovals, and is, we consider, comparable to that. The darker-staining zone appears practically homogeneous, and does not contain, or is not composed of, the fine intensely staining granules characterising the chromatinic zone of the first type of ovals. The comparative extent of the central pale area and the surrounding darker region varies greatly in different individuals. In some the central area is small and the dark zone thick and broad (figs. 65, 67, 68); in others the paler area is much increased and the peripheral zone reduced to a narrow band (figs. 58, 59). Frequently, with this increase of the paler area, the dark-staining substance persists chiefly in the form of two caps, one at each end of the oval, connected only along the two sides of the oval by an extremely thin peripheral layer (figs. 60, 69). Lastly, in a small proportion of ovals, all the protoplasm appears to consist of the darker-staining substance (figs. 64, 70); these may be either small or fairly large.

We have not found such well-marked indications of division in ovals possessing a large area of dark-staining material as in those of the other type; but we are inclined to think that the same transverse binary fission occurs. A not uncommon feature in ovals of this type is the presence of two definite, intensely staining granules, one at the middle of each of the longer sides of the body (figs. 67, 69-71). Frequently these two granules are connected by a fine line, which is sometimes seen to follow the external contour of the body (fig. 70), when it probably represents a very slight annular constriction across it; but at other times the line can be traced with difficulty through the body (fig. 69). It seems probable that these appearances indicate transverse division, but we do not think it occurs to nearly the same extent in ovals showing this second condition of the internal structure.

We may now consider briefly the question of the connection of these different types of form with each other, and of their association together as different phases of one parasite. In the first place, ovals showing the second type of minute structure can be readily connected with those showing the first condition described, by a series of intermediate stages. All degrees in the thinning out of the darker staining area until it is little more than a narrow peripheral ring (as in fig. 59) can be found; and from such a stage to that shown by the individuals, for instance, of figs. 39 or 40 is a very slight transition. Another marked transition stage is seen in fig. 42, where the narrow peripheral, intensely staining zone is slightly thickened around one end; such a condition is manifestly closely connected with that showing a cap of dark-staining substance at each end (as in figs. 60, 61). It is a little difficult to know what interpretation to assign to this darker-staining part of the protoplasm, as found in the second type of oval. In the ovals with a well-marked, finely granular peripheral layer, or with more conspicuous granules, we consider that this zone comprises the chromatinic material of the cell, just as in the case of the crescents. Are we, then, to regard the more or less homogeneous, darker staining

region, when present, as representing chromatin or some allied substance diffused in an extremely fine condition throughout a relatively large area of the protoplasm?

Secondly, as regards the first type of ovals and the crescents, there are several reasons for concluding that these are only distinct phases of one parasite. There is the fact that, on all occasions save one, we have found the two forms associated, and possibly in the case of the third goat ovals may have been present, but were so scarce in comparison with the enormous number of crescents that we did not notice them. Important points of agreement between the two types as regards appearance, one manner of movement, structure, the occurrence of crescents without a flagellum, and so on, have been already dealt with. Lastly, in the case of some individuals, it is purely a matter of choice whether to regard them as bean-like crescents, or as bean-like ovals; thus the form drawn in fig. 38 is readily derivable, one may reasonably suppose, from an aflagellate crescent such as that of fig. 36, while on the other hand, between the parasite of fig. 46 and the oval of fig. 45 there is equally little difference.

Concluding, then, that the above-described different forms all belong to one parasite, it still remains a matter of uncertainty what is the order of transition between them, respectively, and how the different phases should be combined into one life-cycle; it appears very probable that the crescents can give rise to ovals; but we have no indications as to whether the ovals become crescents. Further, we are inclined to the view that the second type of ovals pass into the first type, rather than vice versâ.

The Nature and Affinities of *Selenomastix ruminantium*.—It will be apparent from the foregoing account that this new parasite does not fall readily into any of the known groups of organisms included under the designation Protista; in many respects it is an altogether new type of organism. On first seeing the living, active crescents, with their conspicuous, long flagellum, we naturally thought we had to deal with a new member of the flagellates, as, indeed,

was Certes' opinion originally. From a further study of *Selenomastix*, however, we feel at present very doubtful whether it is a true flagellate. Supposing for the moment that it is, the question of the orientation of the body is a very important one, because this determines, of course, the nature of the division. We have not the slightest indication that the middle of the concave side—approximately the point of insertion of the flagellum—represents the anterior end; on the contrary, such evidence of antero-posterior polarity as we have obtained points to this being in the direction of the longer axis of the body, both in the crescents and the ovals. Hence we must regard the division as transverse. Apart from the entire order of the Dinoflagellates, there are scarcely any Flagellates in which division is transverse. We can find no hint whatever of Dinoflagellate characters in *Selenomastix*. In a crescent which is not commencing to divide, there is neither a second flagellum nor any sign of an annular, transverse groove. It is equally difficult to see any indication of relationship among the Euflagellates. For one thing, the peculiar scattered or diffuse condition of the chromatinic substance is very different from the definite nucleus which is typical of flagellates. Another point which in our view weighs very much against the flagellate affinity of this new creature is the conviction we have that it is capable (either in the crescent or the oval phase) of moving by means of its body alone, somehow, independently of the flagellum, when this is present. We have next to search, therefore, among the vast assemblage of organisms collectively known as bacteria for a clue to the relationships of *Selenomastix*.¹

¹ So far as the ovals alone were concerned, we did not overlook their possible connection with some of the Saccharomycetes, such as *Schizosaccharomyces*. Thanks to the kindness of Dr. Harden, of the Lister Institute, we have been able to compare the ovals with organisms of this group, and it was at once apparent that with them they have nothing whatever to do. Further, the ovals do not resemble in any way another yeast-like type of organism, namely *Blastocystis*, which has been lately described.

We have considered it useful to discuss the possible relationship of *Selenomastix* to the flagellates, because not only does our parasite differ in many respects from any bacterial protist of which we have knowledge, but it also appears to incline more to the Protozoa in one or two important features. In the first place, the host of ordinary bacteria may be at once dismissed from consideration. Dr. Ledingham has kindly looked at the parasites and entirely agrees with us in this opinion; moreover, as above mentioned, cultures made on various media were quite unsuccessful. The most striking feature of *Selenomastix*, from a bacterial point of view, namely, the presence of a large flagellum, easily visible in life and by ordinary staining methods, is only met with, so far as we are aware, in the case of one or two very large spirillar forms and among certain "Sulphur-Bacteria" (*Rhabdomonas*, *Ophidomonas*), of which a good account has been given by Bütschli ('Arch. Protistenk.,' vol. i, 1902, p. 41). The spirillar forms and *Ophidomonas* have a flagellum at each end, *Rhabdomonas* a single, terminal one. In these forms, too, the flagellum shows a tendency to split up, in a somewhat similar manner, into fibrils of varying thickness. These forms have also a well-marked envelope (periplast), which stand off well from the body in many cases; that of *Rhabdomonas* is spirally striated, a point which we have never seen in *Selenomastix*.

It is possible that the origin of *Selenomastix* is to be sought amongst this type of organism; we have had it tested for the presence of sulphur, however, with entirely negative results. Moreover, *Selenomastix* certainly appears very far removed from the spirillar type as generally recognised. Taking first the points of agreement, there is, of course, the transverse division and the absence of a definite, constituted nucleus. Another feature which is somewhat against the Protozoan character of our parasite is the peculiar homogeneous appearance of the protoplasm; the ordinary true flagellates, for instance, which occur in the rumen, look very

different, with their granular cytoplasm. In this respect *Selenomastix* agrees with many bacteria, though it so happens that spirilla, especially the larger ones, are often distinctly granular and sometimes exhibit a chambered structure, of which there is no sign in this new form.

The principal differences from the spirillar type are as follows: There is no true spiral form of the body, or indication of spiral movement. In some individuals, however, there is a hint of a twisting of the axis towards one end, which may or may not represent a permanent condition. There are no terminal flagella, but a single more or less median one. There is distinct evidence of antero-posterior orientation. On the other hand, we have never observed the reversal of direction characteristic of spirilla. Another important distinction is that both the crescents and the ovals can move by means of the body alone, resembling a *Spirochaete*, with which, however, *Selenomastix* has assuredly nothing else in common. Again, individuals certainly vary in width as well as in length; broadly speaking, the larger individuals are both longer and wider than the smaller ones; this is apparent from our plates. Last, but not least, no spirilla of any kind hitherto described, so far as we know, have any phase connected with them comparable to the ovals of *Selenomastix*.

With regard to the vexed question of plasmolysis, we are inclined to think that this occurs, at any rate in the ovals. In living preparations which have been made for some time (whether diluted with a drop of normal saline solution or not), a small proportion of the ovals show a vacuole, or space-like appearance near one or both ends; this is most probably due to the shrinkage of the protoplasm away from the envelope. When the parasites are placed in 5 per cent. or in 10 per cent. salt solution, a somewhat larger proportion of the ovals show this shrinkage appearance, and it is evident, also, here and there in a few crescents. Many of the ovals, however, and the great majority of the crescents—chiefly those, we think, in which, if stained, the envelope would not stand off

in a marked manner—do not appear to be altered at all. They do not swell up, burst, or undergo any other obvious change. An interesting fact, moreover, which we noticed was that, when such preparations were looked at again the next morning, there did not appear to be as many ovals showing the contracted protoplasm as there were soon after the preparations were made. It seemed to us as if the protoplasm must have expanded again in some individuals, which could not, therefore, have been dead.

Of one thing we are sure, namely, that *Selenomastix* does not undergo what the Germans term "Präparationsplasmolyse." This is evident from our figures, which give a fair assortment of the various appearances seen in the stained preparations. Lest it might be thought that the condition in which there are one or two deeply staining masses (regarded by us as chromatinic) in the cell, represents such an artifact, we may point out, first, that there is no larger proportion of such individuals on "dried" Giemsa smears than occurs in properly "wet-fixed" films, made as soon after removal from the rumen as possible; secondly, that every transition can be traced in the development of this phase where the chromatinic substance is compacted into few masses, through conditions where there are a varying number of smaller, but quite prominent granules; and finally, if it were an artifact, the ovals with the first type of minute structure, closely comparable to that of the crescents, might be expected also to show it, which is never the case.

We have now, we think, considered exhaustively the possible directions in which to look for the origin and affinities of this remarkable parasite, so far as we have been able to do so from the facts we have learnt with regard to it up to the present. To sum up, it appears to be entirely unconnected with the Dinoflagellates; it may possibly be derived from some large spirillum, or from an *Ophidomonas*- or *Rhabdomonas*-like form, although we are very doubtful upon the point. For our own part, we are inclined to hazard the suggestion that if there is such a thing as a Pro-Protozoan or

Pro-Flagellate, *Selenomastix ruminantium* (Certes) represents such a Protist, because of the fact that it exhibits certain characters which are common to the flagellate Protozoa, but which are rarely or never possessed by bacteria.

THE LISTER INSTITUTE,
July, 1913.

SUMMARY.

(1) This paper describes a new type of parasitic Protist, to which we have given the name *Selenomastix ruminantium* (Certes). Its habitat is the rumen of Ruminants, especially that of the goat.

(2) The organism occurs in two chief forms—crescents and ovals. The crescents present a homogenous, non-granular appearance, and possess a definite envelope; a single, large flagellum, conspicuous in life, arises from about the middle of the concavity of the crescent. The method of movement is variable; while the movement is sometimes effected by the flagellum, in other cases, perhaps more usually, it is produced by the body alone. In forward progression distinct antero-posterior polarity can be recognised. There is no properly constituted nucleus, the chromatin being present in the form of a peripheral layer, in which granules of varying size may occur, or there may be one or two large masses projecting into the cytoplasm. Division is by equal binary fission, transverse to the long axis.

(3) The ovals resemble the crescents in general, but they never possess a flagellum, although capable of active movement. They show two types of minute structure: (A) ovals in which the chromatinic substance occurs as a narrow, peripheral layer, with or without granules in it. This arrangement agrees closely with that found in the crescents. (B) Ovals in the protoplasm of which two zones can be distinguished, a central, lighter-staining zone, comparable to the cytoplasm of (A) and of the crescents, and a peripheral, darker area of variable extent. This latter may be chromatinic in nature.

(4) We suggest that the second type of oval gives rise to the first type, and also that the crescent may pass into the first type of oval by the loss of the flagellum. We have no indication whether the crescents may be developed from the ovals or not.

(5) Apparently the only Flagellates from which this organism could be derived are the Dinoflagellates, and, apart from the transverse division, there is no indication of any affinity with this group. Further, the nature of the "nucleus" and the capacity of moving by the body alone make it very doubtful if this parasite is a true protozoan.

(6) *Selenomastix ruminantium* differs in important respects from any known bacteria. It has no affinities with Schizo-saccharomycetes, with Blastocystis, nor with the Spirochaetes. In certain characters it shows a resemblance to one or two large Spirillar forms, or to certain members of the Sulphur-Bacteria (e. g. *Ophidomonas*), but while its derivation is possibly to be sought in this direction, it is, nevertheless, very far removed from such forms. We may have in *Selenomastix* an example of a Pro-flagellate.

EXPLANATION OF PLATES 29 AND 30.

Illustrating the paper by Dr. H. M. Woodcock and Mr. G. Lapage, "On a Remarkable New Type of Protistan Parasite."

[All the figures are magnified 2000 times linear. We are indebted to Miss Rhodes for kindly drawing a few of them.]

Figs. 1-38.—All the figures are of crescents, and are from "wet-fixed" films, stained by iron-haematoxylin.

Figs. 1-19.—Single individuals, of various size, showing different conditions of the envelope and of the chromatinic substance.

Figs. 20-26.—Dividing individuals possessing two flagella, but in which the body does not yet show indications of fission. In figs. 20 and 23 the actual splitting of the flagellum is shown.

Figs. 27, 28, and 31.—Later stages of fission, in which the body is also dividing.

Figs. 29, 30, and 32.—Individuals in which the body is dividing, but of which the flagellum has remained single. One of the daughter-individuals will be aflagellate.

Fig. 33.—A small individual in the very last stage of division.

Figs. 34 and 35.—Small pyriform individuals, probably immediately resulting from fission.

Figs. 36 and 37.—Aflagellate crescents.

Fig. 38.—Beam-like crescent, which may be transitional to an oval.

Figs. 39-71 are of ovals, from "wet-fixed" films, stained by iron-haematoxylin.

Figs. 39-57.—Ovals with the first type of minute structure.

Figs. 39-48, 54.—Single individuals of various size.

Figs. 49-53, 55 and 57.—Individuals showing different stages of fission.

Figs. 58, 60-62, 64-68.—Single individuals showing the second type of minute structure.

Figs. 59, 61.—Individuals transitional between ovals of the first and second type.

Fig. 63.—Small dividing individual.

Figs. 69-71.—Ovals showing two definite granules at opposite sides, connected by a line or ring (see text). The individual of fig. 71 is apparently beginning to divide.

Figs. 72-82 are of crescents stained by Giemsa.

Figs. 72 and 73.—Single individuals; flagellum normal.

Fig. 74.—Dividing individual; flagella normal.

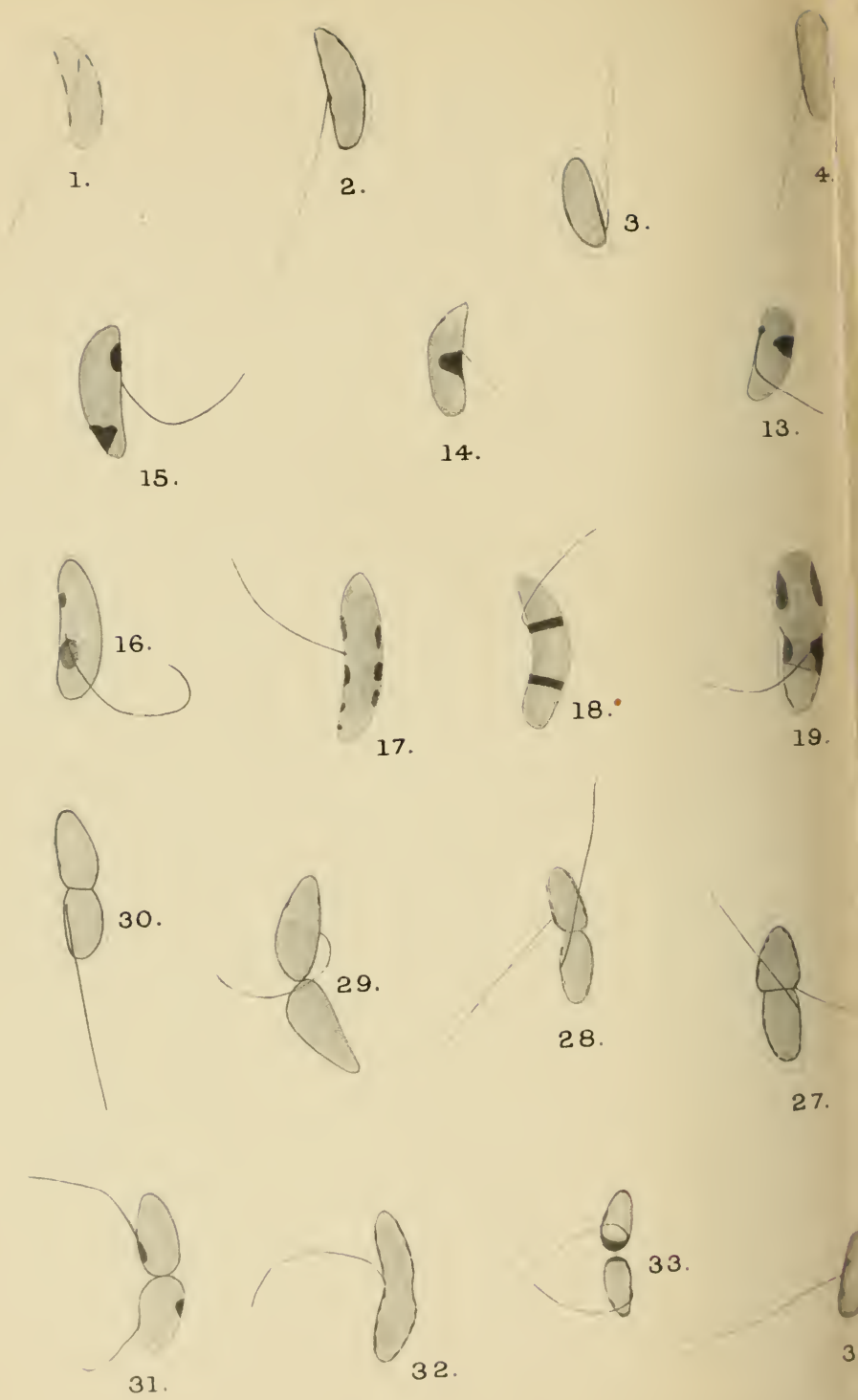
Figs. 75-77.—Individuals showing artificial fraying-out of the flagellum.

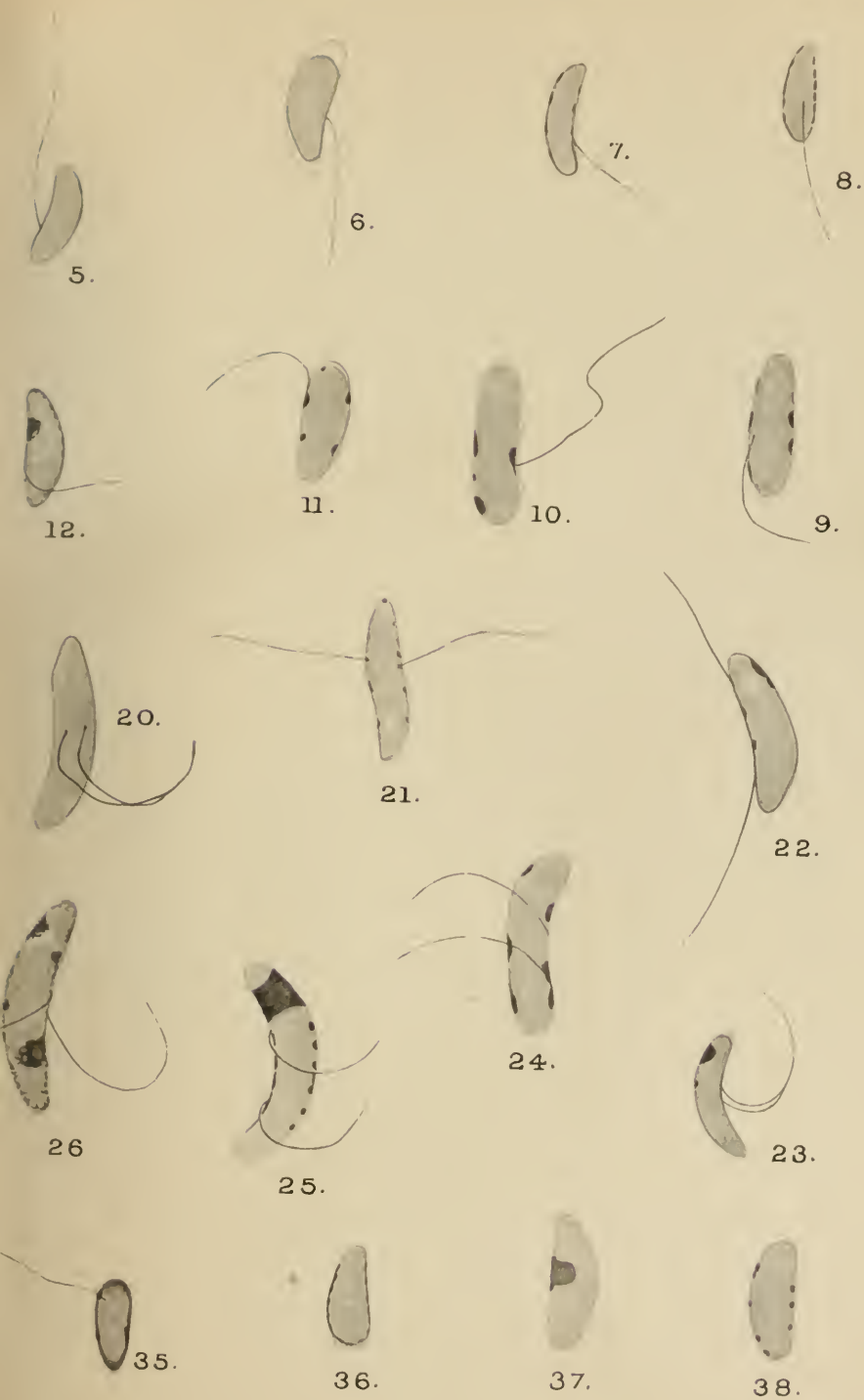
Fig. 78.—Smallest crescent found on a Giemsa smear.

Fig. 79.—Dividing individual, the upper flagellum of which shows indication of fraying-out.

Figs. 80-82.—Aflagellate crescents, showing one or two conspicuous chromatinic masses.

Figs. 83-87.—Ovals stained by Giemsa. In fig. 87 the envelope stands off markedly from the body and shows an annular line (cf. figs. 70 and 71).







39.



40.



41.



42.



54.



53.



52.



51.



55.



56.



57.



58.



70.



69.



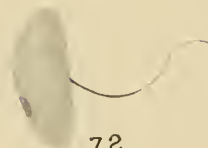
68.



67.



71.



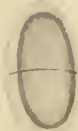
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73.



86.



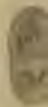
87.



85.



84.



83.



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